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Role of Kidney in Pathogenesis of Experimental Nephrotic Hyperlipemia in Rats.* (21801)

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A condition simulating the nephrotic syndrome of childhood can be produced in rats by the injection of rabbit anti-rat kidney serum (NTS)(1). Marked hyperlipemia develops in the experimental animal, just as in the human disease. In previous work, we have attempted to clarify certain features in the pathogenesis of this rapidly developing hyperlipemia in rats(2-5). The present report describes studies that indicate the essential role of the kidney for the development of this experimental hyperlipemia.

Methods. Nephrotoxic renal disease was produced in Long-Evans and Sprague-Dawley rats by injecting them with nephrotoxic serum (NTS) obtained from rabbits. The details of this technic and the analytical methods used in the blood chemistry studies were previously described(1). Nephrectomies were performed under ether anesthesia using a posterior extraperitoneal approach. Food and water were withheld postoperatively for 48 hours after bilateral nephrectomy.

Results. A. Effect of bilateral nephrectomy. Bilateral nephrectomies were performed on 29 rats. Seventeen of these animals were immediately thereafter injected intravenously with NTS, and 12 others were injected with normal rabbit serum. Sixteen unoperated rats were also injected with NTS. Serum cholesterol and total lipid determinations were done on blood samples obtained 48 hours after the serum injection in these animals, and also on blood samples from a group of 23 unoperated, non-injected normal rats. Control and operated rats were paired to receive similar doses of the same batch of NTS.

The serum total lipid values in these 4 different groups are compared in Fig. 1. Since the serum cholesterol values paralleled those for total lipid, they are not included in the figure. The unoperated rats who were injected with NTS ("N.T.S. Alone") had a very high mean serum total lipid content after 48 hours. In contrast, the rats that had both kidneys excised prior to the injection of

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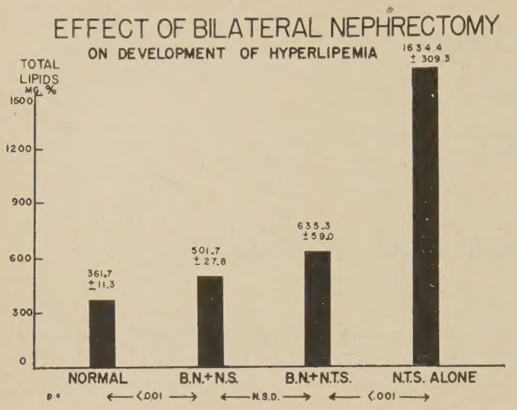

FIG. 1. Effect of bilateral nephrectomy on development of hyperlipemia. Figures above columns are mean values ± stand. error of mean.

Abbreviations: Normal = unoperated, non-injected rats. B.N. + N.S. = rats injected with normal rabbit serum immediately after bilateral nephrectomy. B.N. + N.T.S. = rats injected with NTS immediately after bilateral nephrectomy. N.T.S. alone = unoperated rats given NTS. P = probability that a difference between the 2 columns (indicated by the arrows) as large or larger than that observed could occur by chance. When $p < .01$, a significant difference is considered to exist. N.S.D. = no significant difference ($p > .05$).

NTS (B.N. and N.T.S.) showed a significantly lower total lipid level. This level was no different than for the control group (B.N. and N.S.) who were bilaterally nephrectomized and injected with normal serum rather than with NTS. It has been previously shown(6) that removal of both kidneys in itself results in a slight hyperlipemia comparable to that noted in these studies.

B. Effect of unilateral and bilateral nephrectomy in rats with well-established renal disease. A right nephrectomy was performed on 8 rats with well-established nephrotoxic renal disease. Three to 4 weeks later the other kidney was excised. Blood samples were obtained before and 48 hours after each nephrectomy, and were analyzed for serum cholesterol, total lipid and protein content. The results in one rat (#1372) are illustrated in Fig. 2, and are representative of the findings in the other 7 animals. A decrease in total lipid and cholesterol levels occurred after each nephrectomy in all 8 rats, but the values did not return completely to the normal range.

C. Studies in rats with one normal and one diseased kidney. In 72 rats the left renal pedicle was clamped, as described by Koletsky and Gustafson(7) for 25-30 minutes after the intravenous injection of NTS. Since heteronephrotoxic antibodies are known to be fixed rapidly to renal tissue(8), it was presumed that the injected NTS would be confined in its effect to the unprotected right kidney. Out of the 72 rats, however, only 28 were found to have valid unilateral renal disease as judged by histological examination. Thirty-three additional rats were found to have histological evidence of bilateral renal involvement, and the final 11 rats showed no evidence of renal disease on either side. A control group of 15 normal unoperated rats were injected with the same batch of NTS and killed at time intervals comparable to those for the operated rats. Fig. 3 presents the mean total lipid concentrations in the rats with unilateral ("Unilat.") and bilateral ("Bilat.") renal disease, and compares them with normal control rats ("Normal"), and non-operated rats who were injected with NTS ("N.T.S. Alone"). No significant difference existed between the operated rats who developed bilateral renal disease and the unoperated controls injected with NTS. The group of 28 rats that developed unilateral

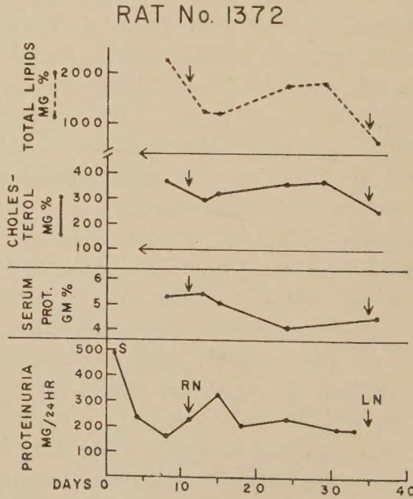

FIG. 2. Effect of 2 stage nephrectomy on hyperlipemia in rat #1372. Horizontal arrow indicates upper limit of normal range of values. S = time of injection of NTS. RN = time of right nephrectomy. LN = time of left nephrectomy.

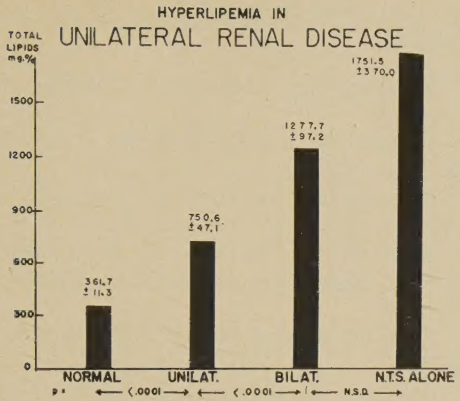

FIG. 3. Relation of hyperlipemia to the presence of one or 2 nephrotic kidneys. UNILAT. = rats who developed disease in right kidney only. BILAT. = rats with bilateral renal disease who were treated similar to previous group. See Fig. 1.

disease, however, had a significantly lower mean total lipid level than the group with bilateral disease, but also had a level that was well above the normal range. The severity of the disease in the group with unilateral involvement was milder than in the group with bilateral disease as judged by the degree of proteinuria, hypoproteinemia, azotemia, and edema.

The right kidney, *i.e.*, the unclamped, disease kidney, was removed in 7 rats with unilateral involvement, and in 17 rats with bilateral involvement. This was done 10-14 days after NTS had been injected. Blood for total lipid and cholesterol determinations was obtained by cardiac puncture 2-3 days before and 48-96 hours after nephrectomy. The mean values obtained in these 2 groups are compared in Table I. Removal of the right kidney in rats with unilateral disease resulted in disappearance of proteinuria and return of serum lipid and cholesterol values to normal. In rats with bilateral renal disease, right nephrectomy resulted in only a slight decrease in these values which did not return to normal.

Discussion. Pressman(8) found that heteronephrotoxic antibodies localize in liver, spleen and lungs, as well as in the kidneys. The hyperlipemia thus could be the result of the action of NTS on tissue other than the kidney. The present experiments, however, indicate that the hyperlipemia is primarily renal in origin, since bilateral nephrectomy prevented its development in rats subsequently injected with NTS.

A correlation between the severity of the hyperlipemia and the quantity of diseased renal tissue was indicated by the finding that hyperlipemia was decreased progressively by removing one and then the other kidney. In accord with this interpretation, also, are the findings in the rats with unilateral renal disease. The level of hyperlipemia was less in these rats than in rats with bilateral disease, and the removal of the diseased kidney abolished the hyperlipemia. In contrast, removal of one of the involved kidneys in rats with bilateral disease produced only partial lowering of the blood lipid level.

These findings suggest the possibility that a "hyperlipemia-inducing" substance is secreted by the nephrotic kidney. The absence of a "lipemia-depressing" agent in the diseased kidney might be considered as an alternate hypothesis. This latter concept is unlikely, however, in light of the findings in the group with unilateral renal disease. Hyperlipemia was produced by the injection of NTS in this group, which would not have been expected if the one normal kidney had been producing a "lipemia-depressing" agent.

TABLE I. Effect of Removal of Right, Nephrotic Kidney in Rats with Unilateral and Bilateral Renal Disease. Mean values ± standard error of mean.

	Unilateral disease		Bilateral disease	
	Before right nephrectomy	After right nephrectomy	Before right nephrectomy	After right nephrectomy
Proteinuria (mg/24 hr)	100 ± 6	14 ± 2*	195 ± 46	156 ± 13
Total lipids (mg %)	566 ± 39	398 ± 11*	1477 ± 108	1000 ± 62*
Cholesterol (mg %)	143 ± 9	97 ± 4	286 ± 20	225 ± 15†

* Indicates significant change over pre-nephrectomy value ($p < 0.01$).
† *Idem* ($p < .02 > .01$).

Summary. 1. Bilateral nephrectomy prevented the development of hyperlipemia in rats injected with NTS. 2. The degree of hyperlipemia in rats with chronic nephrotoxic renal disease was decreased progressively with the removal of each kidney. 3. Production of unilateral renal disease in the presence of one normal kidney did not prevent the development of hyperlipemia. The lipemia was less, however, than in rats with bilateral disease. 4. When the right nephrotic kidney was removed from rats with unilateral renal involvement, the serum cholesterol and total lipid values and the urinary protein content decreased to normal. Right nephrectomy in rats with bilateral renal disease, however, resulted in only slight dcreases in these values which remained above the normal range. 5. It is suggested that a "hyperlipemia-inducing" agent may be secreted by the nephrotic kidney.

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Apparent Reversible Influence of Cosmic-Ray-Induced Showers upon a Biological System.* (21802)

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Like many other animals and plants, the fiddler crab possesses rhythms of primary solar frequency which persist indefinitely without alteration of their phase relationships relative to the outside day-night cycles even when kept under conditions of constant illumination and temperature. Persistent rhythms of primary lunar frequency, with properties similar to those of the daily, seem also very widespread(1). The frequency of these persistent cycles is independent of temperature over a wide range(2-5). The evidence indicates these rhythms to be at least in some measure endogenous(6). Nonetheless, the precision over long periods and the temperature independence makes it probable that some external cues of daily frequency are received by the organisms and serve to maintain the proper mean frequency of the biological cycles. Supporting this latter view is the observation of Brown(7), that the persistent tidal rhythm of activity of oysters away from tidal changes gradually shifted its maximum from times of high tides to the approximate times of lunar zenith and nadir. Also, it has been shown that the metabolic rate of several organisms shows a statistically significant hourly correlation with the direction and rates of barometric pressure change (8).

With these considerations in mind the following work was planned and executed. If cosmic-ray-induced showers are capable of exerting any measurable physiological effect upon the organism, then these might be important factors in maintaining the precision of the endogenous rhythms of organisms since cosmic radiation is known to be rhythmic (9,10). The authors are deeply indebted to Dr. M. Forro for invaluable suggestions and criticisms made throughout the course of the

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TABLE I. Effect of Alteration in Intensity of Showers on *Uca* Black Chromatophores.

		Avg stage of melanophore				
Series	Dates	Lead cover	Control (wood)	Lead minus wood	Total observations†	Total groups†
I	May 25–30	3.95	3.83	+.12	860	43
II	29–31	3.58	3.49	+.09	480	24
III	June 2– 6	3.86	3.74	+.12	500	20
IV	18–21	3.59	3.51	+.08	3625	145
V	28	2.57		+.23	260	13
		2.56*	2.34	+.22		
VI	29	4.26*	4.02	+.24	180	9
			All experiments	+.110 ± .0195		

* Unlike for the other instances which were held under a constant 1.8 cm of lead, these were subjected to fluctuating amounts of lead between 0 and 1.8 cm, the lead being added or removed at the rate of about .3 cm per hr.

† For each, experimental and control animals.

work and to Dr. H. T. Davis and Dr. M. Dwass for advice regarding the statistical analysis of the data.

Materials and methods. The experiments consisted of determining the influence of increase in showers effected by a lead screen on the pigmentary-effector system of the fiddler crab, *Uca pugnax*. This system was chosen because simple, rapid, quantitative methods of study were available, and the system was well-known to exhibit under constant conditions unequivocal rhythms of primary solar and primary lunar frequencies. This chromatophore system, also, reflects very sensitively secretion by at least 2 endocrine sources of the crabs. The experiments were conducted over a 3-month period and involved more than 16,000 individual observations. Some of the experiments were performed in Evanston, Ill., and the remainder at Woods Hole, Mass. In each place some of the experiments were performed in photographic darkrooms and the others in the laboratory, under carefully controlled illumination. White enamelled pans, 7 inches in diameter, each containing 20 or 25 crabs, were placed under 12 x 24 inch lead sheets encased in black cloth, while duplicate random samples, treated in exactly the same manner were placed under wood of the same dimensions as the lead and similarly enclosed in a black cloth. Both lead and wood were supported 5 inches above the crabs in the bottoms of the pans. The state of the melanophores was assayed(1) at intervals over periods ranging from a few hours to several days. Five indicated fully dispersed pigment, one fully concentrated, with 2, 3, and 4 describing intermediate states. In nearly every series the animals were exchanged once between the wood and the lead to reduce the possibility that differences in crab samples were responsible for the results obtained. Data were discarded for all instances in which the control crab showed a chromatophore stage higher than 4.6 or lower than 1.4 since a difference between experimental and control crabs due solely to probability would be expected to increase as the limiting values, 5 and 1, were approached. This difference was conspicuously confirmed by an analysis using pairs of controls on the one hand, and pairs of experimentals on the other, which were assayed simultaneously. This difference due to chance was very small compared with the influence of the lead in the range of stages 1.4 to 4.6 and, in fact, would be expected to operate in a direction predominantly opposite to that of the differences obtained in the experiments to be reported. For most experiments the thickness of the lead was either 1.8 or 2.1 cm; in other series, thinner lead sheeting was used, or, the thickness was gradually altered with time. As described by the well known Rossi curve, by increasing lead thickness one may produce up to a 50% increase in coincidences per hour by cascade multiplication as the thickness increases to about 2 cm. With thicker lead there is a decrease in the coincidences(11).

Results. The experiments and principal results are summarized in Table I and Table II. The first 3 series were obtained in Evans-

TABLE II. Effect of Alteration in Shower-Intensity upon Black Chromatophores of Light-Reversed *Uca.*

	Avg stage of melanophores Lead cover					
Dates (July 19-21)	.3 cm	1.8 cm	Control (wood)	Lead minus wood	Total observations†	Total groups†
9 a.m.-5 p.m. (physiol. 11 p.m.-7 a.m.)	2.71		2.79	−.08	520	26
		2.76		−.03		
				mean = −.055 ± .096		
*9-10 a.m. & 3-5 p.m.	3.00		2.99	−.01	280	14
		3.07		+.08		
				mean = +.045 ± .077		
*11 a.m. & 3-5 p.m. (physiol. 1-4 a.m.)	2.37		2.53	−.16	240	12
		2.41		−.12		
				mean = −.142 ± .118		

* Since these animals have their cycles moved forward about 10 hr, 11 a.m. to 2 p.m. represents the physiological times 1 to 4 a.m. and 9-10 a.m. and 3-5 p.m. represent the physiological times 11-12 p.m. and 5-7 a.m., respectively.

† For each, experimental and control animals.

ton, Ill. The observations were chiefly between 8 a.m. to midnight, with only a few made in the 5 to 7 a.m. hours. The thickness of the lead was 2.1 cm. The interchange of the animals between the lead-covered site and the wood-covered one occurred once a day. The average chromatophore stages for the control crabs and for the experimental ones for each hour of observation for the 3 series are illustrated in Fig. 1A. It is apparent that 19 out of the 20 hours of the day when dispersion of the melanin was determined, the dispersion under lead was equal to (5 hourly periods) or greater than (14 hourly periods) that of the controls.

The difference between the average chromatophore stage in the animals under lead and under wood was essentially the same for the 3 series. The positive sign indicates the crabs under lead to have more dispersed melanin than the control crabs.

Series IV-VI were obtained at Woods Hole. Series IV was conducted more systematically than the preceding ones, and an equal number of observations was made at each hour of the day from 5 a.m. through midnight. During the 4 days each group of animals was exchanged twice between the wood and the lead. The difference obtained resembled in direction and magnitude the earlier series (Fig. 1B). In this series, for 19 out of the 20 hourly times of observation, the animals under lead had equal pigment dispersion (4 instances) or greater dispersion (15 instances) compared with the controls.

Series V was conducted on June 28 between 2 p.m. and midnight, with fiddler crabs divided randomly into 3 lots. One lot was placed under wood, the second under 1.8 cm of lead, the third under lead initially increasing in thickness, the thickness starting at 0.3 cm and then increasing by 0.3 cm at half-hour intervals until 1.8 cm was reached. Under lead the pigment was distinctly more dispersed (Fig. 1C) than in the controls during the first 3 hours. In this and the succeeding series the person staging the chromatophores was not informed which group was being staged, it being passed to the person making the determinations by a second person.

Series VI involved the adding of lead at the rate of 0.3 cm each half hour until 1.8 cm was added and then removing it at the same rate. The animals under lead had distinctly more dispersal melanin at every time of observation (Fig. 1D).

Series I-VI, which showed a remarkable consistency of results, were all performed within the daily period from 5 a.m. to midnight with as many as 5 experienced persons

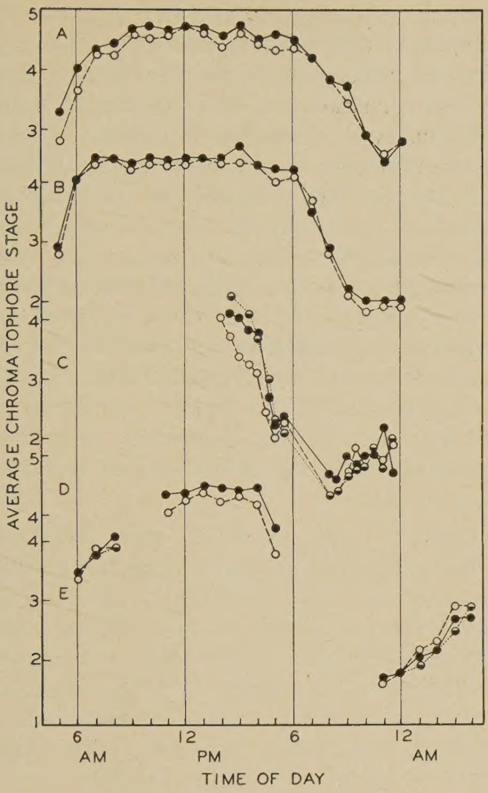

FIG. 1. Comparison of average melanophore stage in animals under lead and controls under wood for various hours in the daily cycle. A. May 25-June 6; B. June 18-21; C. June 28; D. June 29; E. July 19-21. Dashed line—under wood; solid line—under about 2 cm of lead; dotted line—under 0.3 cm or varying thickness of lead.

staging chromatophores. The frequency distribution for the differences between the lead-covered and the simultaneous wood-covered controls (lead-animal value minus the wood-animal value) for the 267 groups of 20 or 25 animals assayed is shown in Fig. 2A. The mode occurs at about +0.20 stage units and the total distribution distinctly favors the plus side. Although a cluster of values occurs near —0.60, the frequency distribution suggests, in general, a relatively homogenous population. A statistical analysis of all the groups of series I-VI showed the mean difference to be +0.110 ± 0.0195.

To learn whether there was any variation in the effectiveness of the showers with the different physiological states of different times of day, it was necessary to procure values obtained predominantly for the hours between midnight and 5 a.m. A large group of crabs was subjected to 3 cycles of illumination by night and darkness by day(12). Judging from color change phases the daily physiological cycles were shifted forward by about 10 hours. These animals were divided into 3 lots and during 3 days each lot was exposed for one day to each, (a) a 1.8-cm lead cover, (b) a 0.3-cm lead cover, and (c) a wooden cover. The observations on the animals were made hourly from 9 a.m. to 5 p.m., which approximated for the animals the physiological times 11 p.m. to 7 a.m. in their normal physiological cycles of color change. It is evident (Fig. 1E) that, unlike for any other time of day, the 1-4 a.m. times in the physiological daily cycle are apparently ones during which the lead cover renders the pigment less dis-

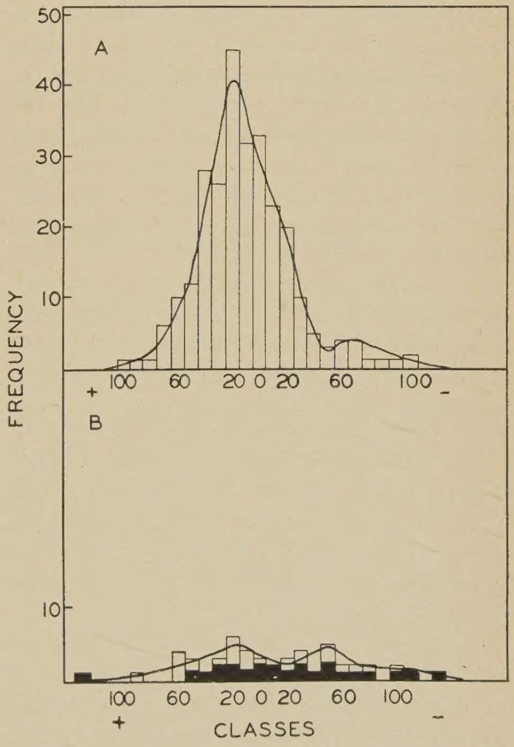

FIG. 2. A. Frequency distribution of differences between various groups of 20-25 crabs under lead and their simultaneous controls under wood for those experiments performed between 5 a.m. and 12 p.m. Positive values indicate greater pigment dispersion under lead. B. Frequency distribution of comparable differences for crabs with reversed daily rhythms observed at the physiological times 11 p.m. through 8 a.m. Shaded area shows values for 1-4 a.m. only.

persed than in controls. The difference between experimental and control values of pigment dispersion (Table II) now were negative, unlike the positive values so uniformly obtained in series I-VI. The frequency distribution for the differences for this series (Fig. 2B) suggests a mode at +0.20 quite as for the first 6 series. In this series, however, there was a much larger percentage of relatively large negative values with these values centering nearer —0.50. There seems to be a clear suggestion of a bimodal distribution though the data are still too few to establish it decisively.

The foregoing analysis suggests that as the melanophores commence pigment dispersing toward the daytime state increased shower intensity results in a lowered degree of dispersion. During at least the greater portion of the remainder of the day the effect is the opposite. When the data for the reversed crabs were analyzed as 2 groups, (a) for the physiological hours 1-4 a.m. alone, and (b) for the physiological hours of 11 and 12 p.m. and 5, 6, and 7 a.m., the former group of hours gave a difference of —0.142 stage unit, and the latter group gave a corresponding difference of +0.045.

An analysis of the significance of the differences for the few available 1-through-4 a.m. observations yielded a mean of —0.142 ± 0.118, or a value not significantly different from zero. The difference between the mean stage obtained in the daytime, +0.110 ± 0.0195 and that obtained for the 4 early morning hours, —0.142 ± .118, gave a difference of 0.252 ± 0.12. There is, therefore, only a reasonable chance that there exists a diurnal rhythm in the response to increased cosmic-ray showers.

Discussion. The results indicate that increasing the intensity of the showers of cosmic ray origin by lead produces a small though significant response of the pigmentary system of *Uca pugnax.* The results suggest, furthermore, the existence of a diurnal rhythm in the character of response of the system to increase in these showers, with a maximum degree of melanin concentration during the early morning hours when the melanophores are normally initiating their pigment dispersion and a maximum degree of darkening of the body or melanin dispersion in the early afternoon when the melanophores are commencing their gradual transition into the concentrated state. This dual type of reaction is suggested in both of the frequency distribution diagrams, Fig. 2, A and B. The 2 modes are quite disproportionate in height in Fig. 2A which includes data predominantly from the day and evening phases of the cycle, but become more nearly equal in Fig. 2B which includes the early morning values in the midst of the 9-hour periods of observation. The diurnal rhythm may be in this case an expression of the response of the particular endocrine glands predominantly active at each of these times of day.

Figge(13) has reported that increased shower production by lead screens of a similar character increased significantly the rate of development of tumors in mice treated with methylcholanthrene. Barnothy and Forro (14) described a strong lethal influence of cosmic-ray shower cascades, obtained by 1.6 cm thick lead plates, upon the unborn and new-born young of mice and rabbits.

There is still no evidence that the capacity to exhibit a response to alteration in intensity of cosmic ray showers is in any way normally operative in the maintenance of the precise 24-hour cycles of the persistent daily rhythm in organisms. However, the variation in intensity of showers investigated here is well within the normal range of magnitudes of variation of the showers normally experienced by organisms.

Summary. 1. Fiddler crabs, of the species *Uca pugnax,* were studied with respect to their capacity to show a response of their chromatophore system to increase in showers of cosmic-ray origin obtained through the interpolation of lead screens. 2. Increasing the showers by lead screen varying from 0.3 to 2.1 cm in thickness and held 5 inches directly above the animals produced significant differences in the response of the pigmentary effector system of the crabs as compared with appropriate controls. 3. There was a strong suggestion of the existence of a diurnal rhythm in the character of the response to in-

creasing showers. The response was increased pigment concentration or body-lightening during the initiation of transition into the day phase of the endogenous daily cycle and increased pigment-dispersion or body-darkening during at least most of the remaining hours of the day. 4. The lead screens, and distances from screen to animals, which were used were responsible for an increase in degree of darkening during the day phase in the cycle (+0.110 ± 0.0195 chromatophore stage units), and for an increase in the degree of lightening during the early morning hours, (—0.142 ± 0.118 chromatophore stage units). 5. A possible functional role of this response to cosmic ray showers is discussed briefly.

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Some Limitations of Bennett's Sulfhydryl Reagent. (21803)

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A new method for the determination of sulfhydryl groups in tissue homogenates has recently been described by Flesch and Kun(1). Their method utilizes 1-(4-chloromercuriphenylazo)-naphthol-2 which was first prepared by Bennett and Yphantis(2) and which was employed by Bennett(3) for the histochemical demonstration of sulfhydryl compounds. Bennett presented considerable evidence to show that the reagent is fixed only by sulfhydryl groups. Flesch and Kun(1) found that glutathione forms a red precipitate with Bennett's reagent and that the amount of precipitate is directly proportional to the amount of glutathione. From these results they concluded that Bennett's reagent should be applicable to the determination of the sulfhydryl content of tissue homogenates. In a later paper Flesch, Golomb, and Satanove(4) explicitly state that Bennett's reagent forms a precipitate with both water-soluble and water-insoluble sulfhydryl compounds. On the basis of the work summarized above, we decided to use the procedure of Flesch and Kun (1) to determine the total sulfhydryl concentration present in Nelson's medium as employed in the *T. pallidum* immobilization test (5). This medium contains glutathione, cysteine, thioglycollate, and, presumably, sulfhydryl compounds extracted from the syphilitic testes.

We were greatly surprised to find that there was no visible reaction between Bennett's reagent and the sulfhydryl compounds present in Nelson's medium. An investigation of the reactivity of Bennett's reagent with individual sulfhydryl compounds and with mixtures of sulfhydryl compounds was therefore undertaken.

Materials and methods. The following compounds were tested: glutathione, cysteine hydrochloride, sodium thioglycollate, ethyl cysteinate hydrochloride, 2,3 dimercaptopropanol, thiosalicylic acid, β-mercaptoethanol, thiomalic acid, and thiourea. Each compound was dissolved in phosphate buffer; the sulfhydryl concentration in each case was 0.01 M. The pH of all solutions at the time of testing was 7.1 ± 0.1. One ml of each sulfhydryl solution was shaken for 20 minutes with 5 ml of a saturated amyl acetate solution of 1-(4 chloromercuriphenylazo)-naphthol-2 (Bennett's reagent). The reactivity of the reagent with mixtures of sulfhydryl compounds was investigated by mixing 1 ml of each sulfhydryl solution with 1 ml of glutathione solution (or 1 ml of cysteine solution) and shaking the resulting mixture with the solution of Bennett's reagent.

Results. Only glutathione, cysteine and thiourea were found to react with Bennett's reagent to give a red or orange-red precipitate. We also found that sodium thioglycollate *prevents* Bennett's reagent from precipitating either glutathione or cysteine. This fact provides an explanation for the lack of reactivity of Bennett's reagent with Nelson's medium. Sodium thioglycollate is not unique in interfering with the procedure of Flesch and Kun. In fact, every non-reacting sulfhydryl compound tested (with the exception of thiomalic acid) interferes similarly. Perhaps thioglycollate and the other sulfhydryl compounds which interfere with the precipitation of glutathione and cysteine preferentially combine with the mercurial, and thus there is not enough of the mercurial remaining to precipitate the glutathione or cysteine.

Summary. An explanation of the reactivity of some sulfhydryl compounds with Bennett's reagent and the lack of reactivity of others is not readily apparent.* It is possible that those sulfhydryl compounds which do not give precipitates with Bennett's reagent form mercaptides which are soluble in the amyl acetate phase. It is obvious, however, that analytical results obtained by the method of Flesch and Kun must be interpreted with great caution.

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* When we communicated our results to Dr. Flesch, he informed us that he had been aware that Bennett's reagent does not react with all sulfhydryl compounds. He was surprised, however, to learn that thioglycollate and a number of other sulfhydryl compounds interfere with the reaction.

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Ca EDTA and the Excretion of Plutonium.* (21804)

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Calcium ethylenediaminetetraacetate (Ca EDTA), a water-soluble chelating agent, has been found to be effective in hastening the excretion of radioactive metals(1-4). This paper reports a series of experiments designed to answer some specific questions that have arisen concerning its effectiveness under a variety of conditions.

Exp. A. Comparison of Ca EDTA with Zr Citrate. One of the more successful approaches for the removal of radioactive metals from the body is Schubert's Zr citrate procedure(5). A comparison of this agent with Ca EDTA was made in the following experiment. In addition, the effect of the combined use of the agents was studied. Four groups of 5 rats (250 g Sprague-Dawley males) each were injected I.V. with tetravalent Pu^{239} in 0.5% Na citrate solution. Each animal received 6 x 10^6 counts/min. Two hours later one group was started on a regimen of 100 mg Ca EDTA, 4 times daily, intraperitoneally and a second group on Zr citrate, 20 mg 4 times daily. The Zr citrate solution was prepared according to Schubert(5). It contained 10 mg/ml of Zr. A third group was injected with both agents simultaneously in the same doses as above. Injections were given for 5 days, stopped for 2 days and then resumed for 3 days. Urine and feces were collected separately and assayed daily. At the end of 16 days the animals were sacrificed and livers, skeletons and remains taken for assay. Urine was assayed by direct plating onto 15 sq cm stainless steel planchets and counting in a windowless methane gas flow proportional counter. Feces were dry-ashed, dissolved in 6 N HNO_3 and assayed in the same manner as the urine.

Results. The effects of treatment are presented in Table I. Both treatments resulted in elevated Pu urinary excretion, the Ca EDTA 14-fold and Zr citrate 10-fold over the controls. These increases were apparently at the expense of fecal excretion, that is, the controls excreted more via this route than did the experimental animals. However, the overall excretion was greater in the treated animals, the Ca EDTA animals 50% and the Zr citrate animals 10% over the controls. The most striking result was the marked shift from skeleton to soft tissue in the Zr citrate animals. The Pu content of the skeletons of these animals was 50% less than the Pu in the skeletons of the controls. The soft tissues of the Zr animals contained 4 times the Pu of the control animals and the liver twice as much.

TABLE I. Comparison of Effect of Ca EDTA and Zr Citrate on Pu Excretion and Distribution.

Tissue	Control	Ca EDTA	Zr citrate	Zr citrate + Ca EDTA
Urine	2.4	33.0	24.2	24.3
Feces	26.3	10.3	7.2	10.7
Skeleton	55.6	40.7	21.1	17.4
Liver	9.6	10.5	19.7	18.0
Soft tissue	7.4	5.4	27.8	30.3

Results are expressed in % of recovered dose. Mean recovery was 96.7 ± 2.5% of injected dose.

The combined treatment produced results very similar to Zr citrate alone. Whatever the combination of Zr citrate and Pu, the binding strength is apparently too great for Ca EDTA to dissociate.

Exp. B. Ca EDTA in chronic Pu poisoning. Previous studies with Pu have dealt primarily with the use of Ca EDTA in situations simulating acute exposures, that is, the administration of the drug was started shortly after the metal was injected. The following experiment tested the efficacy of the drug when given long after the Pu enters the body, using Ca EDTA at first and later Ca EDTA combined with Na EDTA. The later regimen was used to test the hypothesis of Cohen *et al.*(2) that the addition of Na salt would

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* This work was done under the auspices of the AEC.

TABLE II. Fecal and Urinary Excretion in Chronic Pu Poisoning as Influenced by Ca EDTA.

	Urine		Feces	
Day	Control	Experimental	Control	Experimental
0- 7	.88 ± .24	.88 ± .05	7.92 ± .81	14.08 ± .28
8-14	.63 ± .12	.37 ± .07	3.85 ± .76	4.92 ± .28
15-21	.23 ± .07	.26 ± .02	1.84 ± .16	2.86 ± .21
22-28	.28 ± .09	.44 ± .06	1.35 ± .22	1.70 ± .29
29-35*	.46 ± .08	5.10 ± .05	1.19 ± .07	1.79 ± .24
36-42*	.41 ± .06	5.10 ± .10	1.03 ± .13	1.64 ± .19
43-49†	.38 ± .09	4.40 ± .04	1.09 ± .09	1.15 ± .07
50-56†	.35 ± .01	3.14 ± .01	1.01 ± .09	.95 ± .21

Results expressed in % of recovered dose. Mean recovery was 89.85 ± 10.1%.
The ± figures represent stand. dev.
* Animals received 100 mg Ca EDTA, I.P. 4 times daily.
† Animals received 20 mg Na EDTA and 80 mg Ca EDTA, I.P. 4 times daily.

induce decalcification and thereby render deposited radioelements more readily available for removal by chelation. Ten Sprague-Dawley rats, 250 g, males, were each injected intravenously with 4.8 x 10^6 Counts/min. of tetravalent Pu^{239} in 0.5% Na citrate solution. Urine and feces were collected separately and pooled in 7-day batches. Twenty-eight days after the Pu had been injected, 5 of the animals were each injected intraperitoneally with 100 mg Ca EDTA 4 times daily for a period of 2 weeks. Then the experimental animals were started on a course of 20 mg Na EDTA and 80 mg Ca EDTA injected intraperitoneally 4 times daily, a dose level comparable with the previous Ca EDTA regimen. If Na EDTA does facilitate removal of Pu from the body then higher urinary Pu levels than occurred in the Ca EDTA treatment period would result. At the end of 2 weeks of the Na EDTA-Ca EDTA treatment, the animals were sacrificed and tissues taken for assay. The livers, skeletons and remains were dry-ashed, dissolved in 6 N HNO$_3$ and assayed as were the urines.

Results. The data on Pu excretion, both urine and feces, are presented in Table II. During the period of treatment with Ca EDTA, days 29 to 42, there was better than a 10-fold increase in urinary Pu excretion continuously throughout the entire injection period. Fecal excretion was elevated in the experimental group as compared to the controls but this was not related to treatment since the increase occurred primarily in the first week, long before the treatment was started. The data indicate that Ca EDTA did bring forth increased Pu excretion even after the Pu was well laid down in bone. As seen in Table III, this increase was at the expense of all the tissues including the skeleton, *i.e.*, the experimental animals had ¾ the Pu skeletal content as did the controls. Part of the lowered body content of Pu in the experimental animals is due to the higher fecal excretion in these animals but this probably resulted largely in the lowered liver and soft tissue Pu content.

During the treatment involving Na EDTA the urinary Pu was not elevated as compared to the previous treatment period, in fact it was slightly lowered but this lower value corresponds to lower excretion in the controls. From this experiment there does not appear to be any advantage to the addition of Na EDTA to the treatment procedure.

Although this experiment indicated that Ca EDTA would move Pu laid down in bone, it is readily apparent that this is a slow process and prolonged treatment would be necessary to remove a substantial percentage of the body burden. As in every therapeutic regi-

TABLE III. Body Distribution of Pu following Administration of Ca EDTA.

	Controls	Experimental
Urine	4.01 ± .57	21.97 ± .83
Feces	19.04 ± 2.02	29.16 ± 3.5
Liver	13.42 ± 1.72	5.4 ± .62
Skeleton	56.04 ± 2.27	41.32 ± 2.69
Remains	7.37 ± .54	2.30 ± .26

Results expressed in % of recovered dose.
The ± figures represent stand. dev.

men it will be necessary to weigh the inconvenience and hazard of the treatment against the hazard which might result from leaving the Pu in the body.

Exp. C. Ca EDTA and Pu Contamination from Ingestion and by Respiratory Exposure. In view of the fact that Pu is very poorly absorbed from the gastro-intestinal tract(6) it is of interest to know whether parenterally administered Ca EDTA will effect absorption from that site. If so, the use of Ca EDTA might well be contraindicated in cases where appreciable quantities of Pu had been ingested. Likewise, the question arises as to whether Ca EDTA administered parenterally will accelerate transfer of Pu across the lung alveoli and thereby increase the body burden over that which might occur in the untreated cases. The following experiment shed light on these questions. Pu was administered orally by stomach tube in the first study. In the second study the Pu was administered intratracheally by inserting a needle between the rings of the trachea. The procedure was carried out under ether anesthesia. A considerable portion of the dose was lost due to coughing and spewing as the animals came out of the anesthesia. In both studies the administration of 50 mg Ca EDTA was started 2 hours after the administration of the Pu and continued twice daily for 4 days. Excreta were collected and assayed daily. At the end of 4 days the animals were sacrificed and tissues were taken for assay.

Results. There was no essential difference between the amount of Pu absorbed through the gastro-intestinal tract by the control animals and the amount of Pu absorbed by the Ca EDTA treated animals. The only statistically significant difference between the treated and the non-treated animals in the respiratory study is in the Pu content of the carcass which is 1.13 ± .12 in the Ca EDTA treated animals and 3.16 ± .50 in the controls. From these results one might conclude that Ca EDTA did not hasten passage of Pu across the lung membranes into the blood stream but the amount that did come over, about 5%, was excreted to a slightly greater extent in the treated animals.

TABLE IV. Effect of Ca EDTA on Intramuscular Pu Deposits.

	Experimental	Controls	Stand. dev. of diff. of means × 2
Excreta	1.48 ± .17	.46 ± .09	.40
Liver	.46 ± .07	.74 ± .14	.28
Skeleton	1.37 ± .42	.84 ± .30	1.02
Carcass	1.49 ± .52	.75 ± .19	1.10
Inj. hind leg	95.38 ± .72	97.33 ± .27	2.20

Results are expressed in terms of % of recovered dose.

Exp. D. Ca EDTA and Intramuscular Pu Deposits. Tetravalent Pu is slowly resorbed from intramuscular and subcutaneous sites (7). The following experiment was set up to determine if Ca EDTA given intraperitoneally will alter this rate. Ten rats (300 g, male, Sprague-Dawley) were each injected deep into the muscle of the left hind leg with 1.5 x 10^6 Counts/min. of tetravalent Pu^{239} in 1.5 cc of 0.5% Na citrate solution. Two hours later 5 of the rats were each injected intraperitoneally with 50 mg Ca EDTA. Injections were continued twice daily for 4 days. Urine and feces were collected and assayed daily. At the end of 4 days the animals were sacrificed and liver, left hind leg, skeleton and remains were separated and assayed for Pu as described previously.

Results. In Table IV it is seen that the excreta of the experimental group show a significant difference from the controls. The mean of the controls 0.46 plus 0.48 (twice the standard error of the difference of the means) is less than the means of the experimental group, 1.48. None of the other tissues showed a significant difference between the controls and experimental groups. It is of interest that at 4 days there was still over 95% of the injected dose at the injection site. One might conclude that even if Ca EDTA is used one still could debride the major portion of the Pu from a contaminated puncture wound as late as 4 days or later after an accident.

Summary. 1. A comparison of Ca EDTA and Zr citrate for the therapy of acute Pu poisoning was made in which it was found that Ca EDTA brought forth greater Pu excretion (50% greater than controls) than did the Zr citrate (10% greater than controls). The use of the latter agent resulted in lower

Pu skeletal content than occurred in the Ca EDTA treated animals. Combined Ca EDTA and Zr citrate treatment produced the same results as Zr citrate alone. 2. Ca EDTA administered long after Pu was deposited in bone did remove Pu from the skeleton but this is a slow process. 3. A comparison of a regimen of Na EDTA and Ca EDTA with one using Ca EDTA alone in acute Pu exposures showed no advantage to either. 4. Ca EDTA did not influence passage of Pu across the lung alveolar membrane. 5. Ca EDTA did not influence the rate of mobilization of Pu from an intramuscular site. 6. Ca EDTA did not influence the rate of absorption of Pu from the gastrointestinal tract.

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A Glucosamine Conjugate Occurring in Human Urine. (21805)

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The purpose of this study was to measure the normal daily excretion of conjugated hexosamine, and to investigate its chemical nature. While several workers(1-5) have reported the presence of hexosamine in hydrolyzed urine, to our knowledge no further characterization of, or quantitative distinction between, "free" and "bound" glucosamine has been made.

Methods. Assay. Twenty-four hour samples of urine were collected under toluene, measured, and refrigerated. Fifty-ml aliquots were dialyzed against tap water for 24 hours in Visking cellulose tubing of 4 cm diameter. An aliquot of the non-dialyzed portion, usually 10 ml, was removed and one-tenth volume of concentrated HCl added. The mixture was placed on a steam bath for 4 hours, and then taken to complete dryness in a current of air. The pH of the residue in water was adjusted to 9.8 (electrometric) with NaOH and the volume made to 8.0 ml. Duplicate 2.0 ml samples were assayed for hexosamine according to the procedure of Schloss(6). Commercially obtained glucosamine hydrochloride, recrystallized from methanol (m.p. 186-8°, uncorr., decomp.), was used as the standard. The values are expressed as free glucosamine. Galactosamine (chondrosamine) hydrochloride was prepared and purified according to Stacey(7). Both final products were anthrone negative and chromatographically pure, except that each showed traces of the other in the Lut system (see below). In 8 successive determinations on the same urine sample, the results differed from the arithmetic mean by not more than 1.2 mg. Quadruplicate determinations on each of 4 other samples demonstrated the same degree of precision. Nineteen cm strips of Whatman #1 paper were used for ascending chromatography. The solvent systems used were: 2,6-lutidine:water (65:35) (Lut); *n*-butanol:acetic acid:water (4:1:5) (BuAcH_2O); *n*-butanol:acetic acid:water (4:1:1) (BuAc); *n*-butanol:ethanol:water (4:1:1) (BuEt); ethyl acetate:pyridine:water (2:1:2) (EtPyr); and *tert*-butanol:water (75:25) (*t*-BuH_2O). The developing sprays were 0.2% ninhydrin in acetone or M/10 aniline hydrogen oxalate(8).

Isolation of partially purified hexosamine. Thirteen hundred ml of urine was dialyzed

24 hours, concentrated at 50°C *in vacuo* to 200 ml, and dialyzed a further 15 hours in the refrigerator, against distilled water. The contents of the dialysis sac were dried *in vacuo,* 10 ml of HCl and 50 mg $SnCl_2$ were added to the residue and the mixture refluxed on a steam bath for 4 hours. It was then treated with charcoal (Norit A) and filtered. Stannous ion was removed with H_2S and the solution blown dry with air. The residue was treated with 25 ml of methanol:acetone (1:1). A hexosamine negative residue was removed. When a large excess of acetone was added to the filtrate, a hexosamine positive precipitate resulted, which was removed and dried. This material will be referred to as the "unknown."

Results. No hexosamine test was obtained until after hydrolysis. When hourly samples were assayed during hydrolysis, it was found in experiments with 2 urines that 4 hours were required for the complete release of bound hexosamine. A 19-hour hydrolysis did not alter the results. In an experiment in which a value equivalent to 22.8 mg per day was obtained, a further 24 hour dialysis lowered the value to 16.5; a 7-day dialysis gave a value of 12.9. Although the 24-hour figure for bound hexosamine is possibly a few mg low, the 24 hour dialysis appears optimal for removing interfering substances with a minimal loss of hexosamine.

To determine the stability of the conjugate to bacterial action, urine was kept in the refrigerator with and without preservative. In the sample without toluene, the hexosamine fell from 43.1 to 26.7 in 3 weeks. In another urine the original value was 23.8; after 6 days with and without preservative (phenylmercuric nitrate, 10 ml of 1:1000), it was respectively 24.4 and 12.5 mg. Under toluene, an original value of 31.5 mg per day was obtained for another sample, and 32.3 mg 24 days later.

The color obtained with the samples had the characteristic maximum at 530 mμ(9), as determined with the Beckman DU spectrophotometer. One specimen was off-color (orange-red), but exhibited the proper absorption maximum and a normal 24-hour value.

The bound hexosamine excretion in 30 specimens from 14 normal adult males was 32.4 ± 7.2* mg per day (range 20.8—48.2); for 13 adult females (18 specimens) it was 25.9 ± 5.8* mg per day (range 13.1—35.6). The difference between the 2 groups was highly significant ($p<0.01$). The inter-individual variation is approximately the same as that for successive 24-hour samples from the same individual.

In single experiments on a healthy subject, the following conditions did not cause the values obtained to be outside the normal range; three days fasting, three days of carbohydrate-free diet, ingestion of 8 g of sodium salicylate (chemical stress), ingestion of 840 mg of rutin, or ingestion of 4.2 g of ascorbic acid. No correlation between bound hexosamine and age, somatotype, or urinary volume was found. Day and night excretions of bound hexosamine were equal.

Nature of Conjugate. The conjugate in dialyzed urine was not precipitated by acetic acid, lead acetate, or 0.6 M saline. No sulfate, and only a trace of hexuronic acid(12) was found in the unconcentrated dialyzed urine. After a 5-fold concentration, ninhydrin and biuret tests were still negative. The cation exchange resin, IR-120 (Rohm and Haas), took up the conjugate to a small extent.

The dialyzed urine was treated with various crude and crystalline enzymes. In controlled experiments, no measurable hexosamine was released by protease (Takamine), pancreatin (3 X USP, Takamine), trypsin (3 X recryst., Worthington), carboxypeptidase (2 X recryst., Worthington), pepsin (1:10,000, Wilson), cysteine-activated papain (2 X recryst., Worthington), emulsin (General Biochemicals), α-amylase (General Biochemicals), β-amylase (General Biochemicals), lysozyme (2 X recryst., Worthington), hyaluronidase (bovine testicular, Cudahy), and "special diastase 160" (Takamine). The following enzyme preparations, freed the percentage of hexosamine indicated, as compared

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$$* \sigma = \sqrt{\frac{\Sigma d^2}{N-1}}.$$

TABLE I. R_f Values of Ninhydrin Positive Substances in Dialyzed, Hydrolyzed Urine.

System*		
BuAc	Lut	$BuAcH_2O$
.09	.08	.04
.20	.16	.16
.26	.24	.25
.32	.29	.44
.56	.37	.71
.69	.44	
	.61 (brown)	

* See text for key to abbreviation.

with acid hydrolysis: maltase (fungal origin, Bios) 13, brewer's yeast (Sperti) 12, "mylase SA" (Wallerstein) 5, activated crude papain (Riverplate) 17, malt diastase (1:50 in 3 min., Difco) 25, filtered saliva, 16. Controls were run in these experiments. The latter 5 preparations may each have some "maltase" present(11,12).

Ascending paper chromatography of dialyzed urine showed the major saccharide present to be galactose, after hydrolysis according to Evans and Mehl(13). The Rf for galactose in the $BuAcH_2O$ system was 0.20; the unknown had an R_f of 0.19. In the Lut system each has an R_f of 0.57—0.58. Other sugars(4,14) were not present in sufficient amounts for detection. Several ninhydrin-positive substances other than hexosamine are present in relatively small amounts in the hydrolyzate (Table I). The dinitrophenylhydrazones of glucosamine, galactosamine, and the unknown were prepared and chromatographed according to Kent *et al.*(15). These compounds ran too close together for an unequivocal distinction to be made.

Chromatography of the knowns and the unknown, using the lutidine-water system, gave the clearest indication that the hexosamine was glucosamine (Table II).

This was confirmed by oxidizing the unknown with ninhydrin and comparing the oxidation product on paper strip chromatograms with arabinose and lyxose(16,17). The coincidence of the unknown with arabinose showed the unknown to be glucosamine (Table III).

TABLE II. R_f Values of Hexosamines with Lutidine : Water (65:35) System with Ninhydrin Developer.

	R_f	No. determinations	Color
Glucosamine HCl	.41-.45	8	purple
Galactosamine HCl	.21-.23	7	blue
Unknown	.44-.45	4	purple

TABLE III. R_f Values of Ninhydrin Oxidation Products of Hexosamine Conjugate.

	System*			
	Lut	BuEt	EtPyr	$t\text{-}BuH_2O$
Unknown	.53	.24	.68	.57
Arabinose	.57	.27	.69	.60
Lyxose	.61	.32	.75	.66

* See text for key to abbreviations.

Discussion. The conjugate is apparently endogenous in origin, since its excretion is unaffected by fasting or by a carbohydrate-free diet. The latter was tried because dietary glucose may be a precursor of glucosamine (18,19). Its origin is unclear at this time, but a study of the effect of various pathological conditions on the amount excreted may yield some clue, and might be of diagnostic value. A lyophilized sample† of the mucoprotein of Tamm and Horsfall(20) contained 2.5% hexosamine. By this technique, urinary mucoprotein isolated by the procedure of Anderson and Maclean(21) has a hexosamine content of about 3%. While the results presented show the predominant hexosamine in the conjugate to be glucosamine, the possibility of some minor contribution from galactosamine is not excluded.

The enzymatic results, and the presence of a sufficient quantity of galactose(14), suggests that the conjugate is primarily a compound involving glucosamine and galactose. Use of a more nearly pure preparation of maltase, or of some other conditions, might result in more efficient hydrolysis.

Summary. A hexosamine conjugate is excreted by normal adults to the daily extent of 32.4 ± 7.2 mg for men and 25.9 ± 5.8 mg for women. The conjugate originates endogenously, but is unrelated in quantity to somatotype, age, or urinary volume. The hexosamine is glucosamine. There is no evidence that the conjugate is a mucoprotein. It

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† Kindly supplied by Dr. W. H. Royce, Bowman-Gray Medical School, Winston-Salem, N. C.

appears to be principally a combination of glucosamine and galactose.

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Comparison of Strophanthin-K and Tryptamine-Strophanthidin after Intraventricular Injection in Unanesthetized Dogs.* (21806)

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Recently Otto *et al.*(1) published a report on some of the clinical aspects of tryptamine-strophanthidin, a new compound which has a typical digitalis action on the isolated cat papillary muscle and on patients with auricular fibrillation. Tryptamine-strophanthidin differs from the usual synthetic drugs of the strophanthidin series in that it has the tryptamine residue condensed with the aldehyde group in position 10 of the cyclopentanoperhydrophenanthrene nucleus rather than to the hydroxyl group in position 3. Because the available information indicated a high potency for this new drug, we decided to compare its action with that of strophanthin-K after intraventricular injection in unanesthetized dogs.

Methods. Fifteen, normal mongrel dogs of both sexes, weighing 7.8 to 16.1 kg, were anesthetized with sodium pentobarbital, 35 mg/kg, intravenously, and an indwelling third ventricle cannula inserted as described previously(4). The animals were used one week later in the unanesthetized state. All third ventricle injections were made via a polyethylene tube attached to the cannula. The total volume injected per dose varied from 0.2 to 1 ml. Five additional dogs were used for intravenous controls and received their injections via the anterior subcutaneous vein of the foreleg. Electrocardiograms were obtained with a Sanborn Viso-Cardiette using Lead II.

Results. Table I gives a comparison of typical effects produced by the 2 drugs. Intraventricular injection of 6.4 μg/kg of strophanthin-K produced bigeminy and bradycardia while 8.4 μg/kg resulted in an auricular tachycardia. Secondary effects consisted of:

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* This article is based on work performed under Contract No. AT-04-1-GEN-12 between Atomic Energy Commission and University of California at Los Angeles.

TABLE I. Comparison of Effects Produced by Strophanthin-K and Tryptamine-Strophanthidin.

Drug and dose	Time, min.	Pulse rate	Cardiac effects	Secondary effects
Strophanthin-K, 6.4 μg/kg V.C.*	0	95	Normal	In 2 min.: mydriasis, defecation, mucous membrane and retinal pallor, salivation, generalized tremor.
	10	60	Bigeminy	
	20	70		
Strophanthin-K, 9.4 μg/kg V.C.; pentobarbital, 120 mg I.V.*	30	70	Trigeminy	Diaphragm retracted, opisthotonus
	38	160	Alternating ventricular extrasystoles	
	43	160		
	53	160	Sinus rhythm	Anesthetized; no salivation, tremor or pallor. Expired in 3 hr.
Strophanthin-K, 20.7 μg/kg V.C.	0	60		
	2	64	Bigeminal Luciani pauses	Tremor
	4	85	Trigeminy, quadrigeminy with pauses	Salivation, mydriasis, tense abdomen, tremor
	10	100	Accentuated P-waves, inverted T-waves	
	16	110	Alternating ventricular extrasystoles	Lateral nystagmus, opisthotonus, exophthalmos, deep respiration
Pentobarbital, 180 mg I.V.	25	180	Proxysmal ventricular tachycardia	Anesthetized, expired in 7 hr of cardiac failure.
Tryptamine-strophanthidin, 4.4 μg/kg V.C.	0	80	Normal	
	5	65	Bradycardia, rounded ST-segment	Exophthalmos, mydriasis, tremor, opisthotonus
	11	52	Bigeminy, trigeminy	
	13	55	T-waves somewhat flattened	
	27	58	Normal bigeminal beats	Pupils normal, apparent recovery.
Tryptamine-strophanthidin, 14.7 μg/kg V.C.	0	80		
	5	120	Sinus arrhythmia, irregular amplitude of P-waves	Mydriasis
	7	210	Auricular tachycardia	Defecation, urination, salivation
	12	280	Alternating extrasystoles, proxysmal ventricular tachycardia	Vertical nystagmus
Pentobarbital, 120 mg I.V.	20	165	Sinus tachycardia, accentuated P-waves	Anesthetized, animal survived.

* V.C. = Intraventricular injection; I.V. = Intravenous injection.

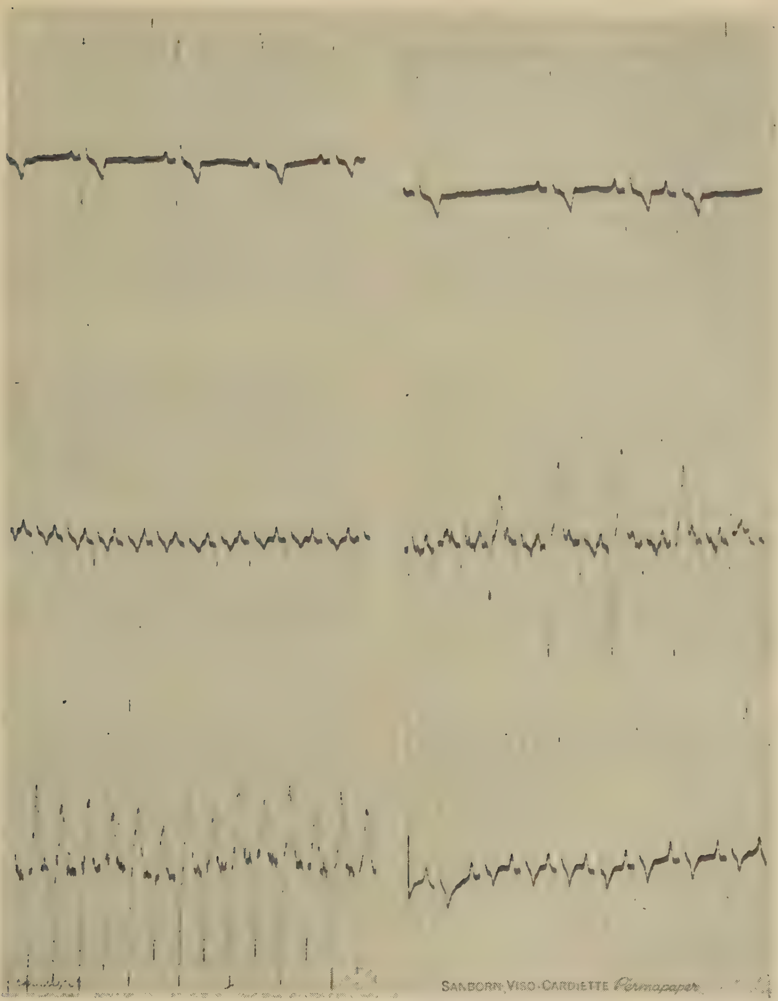

FIG. 1. Tryptamine-strophanthidin, 14.7 μg/kg intraventricularly. Time sequence left to right: 0, 5, 7, 10, 12 and 20 min.

mydriasis, salivation, defecation, shivering, mucous membrane and retinal pallor, and a generalized tremor. Doses of 31.4 to 57.4 μg/kg of strophanthin-K produced ventricular extrasystoles and paroxysmal ventricular tachycardia within 3 minutes. In some animals, sinus tachycardia, periodic P-wave supression, bigeminy, trigeminy, and multi-focal ventricular tachycardia were observed after 2 to 9 minutes. Additional secondary effects were: lateral or vertical nystagmus, barking, generalized excitement, opisthotonus, tense abdominal musculature, tachypnea, nausea, emesis, urination, orbicularis muscle twitching, and death by respiratory paralysis and ventricular fibrillation. These actions must be centrally induced because intravenous strophanthin-K, 31 μg/kg, had no effect on the animals while 77 μg/kg produced a bradycardia of only 9 minutes duration. No secondary effects were observed at either dose.

Tryptamine-strophanthidin, 3.2 to 4.4 μg/kg intraventricularly, produced bradycardia, decreased T-wave, increased Q-wave, and a depressed ST segment typical of digitalis. The secondary effects were similar to those seen with strophanthin-K. The 10.9 and 17.2 μg/kg intraventricular doses of tryptamine-strophanthidin corresponded in their cardiac and secondary effects with those observed with the 31.4 and 57.4 μg/kg doses of strophanthin-K. Intravenous tryptamine-strophanthidin, 8.9 to 9.8 μg/kg, produced little effect on the animals and doses of 20.1 to 26.8 μg/kg produced a biphasic T-wave and a deep Q-wave or T-wave inversion with a late sagging ST segment and a bradycardia, rate 100 decreasing to 75. The cardiac effect had a duration of 10 minutes and none of the secondary effects seen after third ventricle injection were observed. Fig. 1 illustrates a typical electrocardiogram obtained with tryptamine-strophanthidin which is similar to that obtained with strophanthin-K.

The effects observed after third ventricle injection must be related to the drugs because injection of the vehicles used produced no effect in the animals. Furthermore, anesthetization of the dogs with sodium pentobarbital reduced the cardiac rate and induced a regular sinus rhythm.

Discussion. From our results as well as those of Otto *et al.*(1), it appears that tryptamine-strophanthidin produced cardiac effects similar to those seen with the digitalis glycosides. The results obtained indicate that this new drug may be somewhat more potent than strophanthin-K, but further work must be done to firmly establish this point because Chen and Henderson(2) have shown that the aglucone is more potent than tryptamine-strophanthidin. There can be no doubt that both compounds have central actions which affect the myocardium. The rapidity of onset after intraventricular injection does not allow time for sufficient drug to diffuse into the systemic circulation. The type of effects seen from the 2 routes of administration are diametrically opposite. Furthermore, barbiturate anesthesia abolished the arrhythmia (3) and intravenous hexamethonium or chlorpromazine abolished the arrhythmia(4,5). Some of the effects seen after intracerebral injection might be attributed to irritation caused by the strophanthidin compounds. However, quinidine, also an irritant, did not cause the same cardiac effects, indicating at least that these effects are due to specific drug action and not irritation(4).

The bradycardia and typical digitalis changes in the T-wave and the RS-T segment of the electrocardiogram observed with small doses of both strophanthidin derivatives indicate that, although such drugs can be shown to have direct effects on the myocardium, they may also affect this organ via the central nervous system. It is highly unlikely that the bradycardia seen after intraventricular injection can be ascribed to a direct action on the carotid body because intravenous doses from 3- to 10-fold greater were required to produce slowing of the cardiac rate. This appears to be contrary to the report of Heymans *et al.* (6), but his experiments employed anesthesia which we have shown abolishes the central effects of these drugs. However, the short duration bradycardia occurring after intravenous injection could be mediated through an action on the carotid body. The larger intraventricular doses of the strophanthidin derivatives produce all the manifestations of cardiac glycoside toxicity indicating that this action too may have a central component.

Summary. A comparison has been made of the effects produced by the intraventricular injection of strophanthin-K and tryptamine-strophanthidin. Both drugs produce bradycardia and typical digitalis changes in the T-wave of the electrocardiogram at low doses and manifestations of digitalis toxicity at higher doses. Some of the effects were not observed after much higher doses intravenously, thus indicating that the effects seen had their origin in the central nervous system. Anesthesia abolishes these actions of the strophanthidin derivatives, indicating that anesthesia should be avoided in the study of centrally mediated drug effects.

The authors wish to thank Mr. Harry Althouse of Sandoz Pharmaceuticals for the strophanthin-K and Dr. K. K. Chen, Lilly Research Laboratories for the tryptamine-strophanthidin used in this study; and W. G. McCormick, V. Silas and John Bettington for their technical assistance.

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Tissue Electrolyte Content of Potassium and Protein-Deficient Rats. (21807)

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In a recently reported study(1) it was found that when protein was returned to the diet of potassium and protein-deficient rats there occurred a precipitous fall of the skeletal muscle potassium content. The animals continued to excrete potassium unabated despite a positive nitrogen balance. However, the negative potassium balance was found to be insufficient to account for the muscle potassium loss and it was suggested that with protein supplementation there might have been a redistribution of potassium within the body.

It seemed possible that the loss of potassium from the skeletal muscle of potassium and protein-deficient rats following realimentation with protein may be accompanied by an increase of the potassium content of other tissues. The present experiments were therefore undertaken to compare the electrolyte balance and the chemical composition of skeletal muscle, heart muscle, kidney and liver of potassium and protein-deficient rats upon supplementing the diet with (1) potassium and protein, (2) potassium, and (3) protein.

Methods. Young adult male rats of the Wistar strain, weighing between 260 and 290 g, were placed on a control diet(2)* for a period of at least 7 days. They were then divided into 2 groups: One group continued to receive the control diet for 5 weeks, while the second group was placed on a diet deficient in both potassium and protein for a period of 5 weeks. At the beginning of the 5th week, the rats were placed in individual metabolism cages for collections required for the balance studies. At the close of the 5-week period, the animals receiving the control diet and members of the group given the deficient diet were sacrificed to collect samples for the chemical analysis of the blood plasma and the tissues. Other members of the group given the deficient diet were placed for 6 days either on (1) the control diet, (2) a low-protein diet, or (3) a low-potassium diet. The experiment was then terminated and blood and tissues were collected for analysis. The nitrogen intake and the intakes of chloride, sodium and potassium were estimated on the basis of the amount of food consumed and the results of a chemical analysis of an aliquot of the ration. The excretions of the latter elements were determined by chemical analysis of aliquots of urine plus feces previously homogenized in a Waring blendor. The procedure for the collection of the tissues and the methods of their chemical analysis were essentially the same as previously described(2,3), except that plasma, tissues and diet sodium and potassium were determined with the Beckman Flame Spectrophotometer on nitric acid digests.

Results. Plasma composition. The data presented in Table I reveal that aside from a

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* Sheffield "De-Vitaminized" casein was used as the protein source; this brand has a lower potassium content than the casein employed earlier. For this study the vitamin supplements were considerably increased. The majority of vitamins were more than doubled in quantity and p-aminobenzoic acid, folic acid and vit. B_{12} were added to original supplement. The salt mixture as used in the earlier study was modified by increasing the calcium to maintain a Ca:P ratio of between 2:1 and 1:1 whether the diet contained 24% casein or was protein-free. Further, the manganese content was increased, and, the silicon was omitted.

TABLE I. Electrolyte Content of Plasma.

Series*	No. of rats†	Na	K	Cl	HCO_3
		mEq/kilo plasma water			
A	12	158	4.4	106	27
		4.7‡	.3	2.4	2.3
B	7	159	3.6	110	25
		2.0	.5	3.1	3.9
C	3	152	5.0	106	30
		2.3	.5	1.5	0.7
D	3	155	4.4	110	25
		2.7	.1	1.0	1.5
E	9	160	2.5	102	29
		4.2	.4	3.2	1.5

* Series A, control diet 5 wk; B, low-potassium-low-protein diet 5 wk; C, low-potassium-low-protein diet 5 wk, then supplemented with potassium and protein 6 days; D, low-potassium-low-protein diet 5 wk, then supplemented with potassium 6 days; E, low-potassium-low-protein diet 5 wk, then supplemented with protein 6 days.

† Number indicates No. of groups of 2 or 3 rats.

‡ Stand. dev. value is listed below each average value.

slightly lowered plasma potassium concentration, the compositions of the plasma from rats fed the potassium and protein-deficient diet and from rats receiving the control ration were not significantly different. Substituting the control ration for the deficient diet or supplementing the deficient diet with potassium resulted in a restoration of the plasma potassium concentration. However, when the deficient diet was supplemented with protein for 6 days, the plasma potassium concentration became further reduced, and, there was an indication of a tendency toward a lowering of the plasma chloride concentration and a rise of the plasma bicarbonate content. A fall of the plasma chloride concentration and a rise of the plasma bicarbonate content may be anticipated since these are typical findings encountered in potassium-deficient rats(3,4).

Electrolyte content of tissue. The results of the chemical analysis of skeletal muscle, kidney, heart muscle and liver are summarized in Table II. It will be seen that no pronounced differences were found between the chemical compositions of the tissues examined from rats fed the deficient diet and of the tissues from rats maintained on the control diet. Attention should however be called to the slightly higher average values for chloride, sodium and potassium found in heart muscle and in liver from the animals fed the deficient diet. The return of both potassium and protein or of potassium alone to the deficient diet resulted in no marked change in tissue electrolyte concentrations. The small changes produced in skeletal muscle by the deficient diet tended to be corrected by either of the above supplements. Feeding the control diet tended to correct the slight changes in the liver. Neither supplement altered the slightly elevated concentration of electrolytes in kidney and heart tissues. Significant alteration of the electrolyte composition of the examined tissues was found when the deficient diet was supplemented with protein. Thus, as was noted previously(1), during a 6-day period the skeletal muscle potassium concentration decreased from an average of 42.7 mEq per 100 g of fat-free solids to 32.9 mEq. Simultaneously, the muscle sodium concentration increased from 11.0 mEq per 100 g of fat-free solids to 14.4 mEq. Further, when compared with the average figures found at the conclusion of the 5-week period of feeding the potassium and protein-deficient diet, the supplementation of the deficient diet with protein

TABLE II. Average Electrolyte Content of Tissue.*

Series†	Skeletal muscle				Kidney				Heart				Liver			
	H_2O	Cl	Na	K	H_2O	Cl	Na	K	H_2O	Cl	Na	K	H_2O	Cl	Na	K
	(g)	(mEq)			(g)	(mEq)			(g)	(mEq)			(g)	(mEq)		
A	322	5.1	9.3	44.2	341	20.6	25.1	34.1	336	9.8	17.7	35.6	257	9.2	9.1	35.0
	(6)‡	(6)	(6)	(6)					(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)
B	329	6.4	11.0	42.7	355	21.6	26.3	34.9	360	12.0	20.5	37.3	310	12.0	12.3	37.8
C	333	5.4	9.3	46.1	356	23.3	28.7	35.4	366	12.0	20.6	38.5	253	10.2	9.4	35.7
D	328	6.0	9.0	47.1	359	22.1	27.5	37.4	365	12.2	20.0	37.9	306	12.5	11.6	37.6
E	326	5.4	14.4	32.9	353	19.0	26.0	30.6	356	10.9	21.0	33.9	272	9.4	10.9	34.6

* Results of chemical analyses are expressed/100 g of fat-free solids.

† Compare with Table I.

‡ Numbers within parentheses indicate No. of animals, where the number is different from that shown in Table I.

TABLE III. Average Electrolyte and Nitrogen Balances.

	Depletion period† Balance/rat/day				Supplementation period Balance/rat/day			
Series*	Cl, mEq	Na, mEq	K, mEq	N, mg	Cl, mEq	Na, mEq	K, mEq.	N, mg
A	.21 (3)‡	–.10 (3)	.10 (3)	48 (3)				
B	.25	.0	–.05	–39				
C	–.02	–.13	–.05	–45	.15	.24	.65	347
D	.07	–.19	–.05	–45	–.32	–.32	.01	–39
E	.17	.19	–.09	–56	–.10	–.26	–.06	104

* Compare with Table I.

† The fifth wk of the 5-wk dietary period.

‡ Numbers within parentheses indicate No. of animals, where the number is different from that shown in Table I.

resulted in a decrease of the average potassium concentration of kidney, heart muscle and liver. From these data, it will be evident that the pronounced fall of the skeletal muscle potassium was not accompanied by an increase of the potassium concentration of kidney, heart muscle or liver.

Balance studies. Data relative to the balances of nitrogen, chloride, sodium and potassium for the fifth week of a 5-week dietary period and for six days of supplementation are presented in Table III. The chloride and sodium contents of all diets were uniform and approximately equal. The average daily intakes per rat of electrolytes and nitrogen were as follows: Control diet, Na and Cl, 3.8 mEq, K, 1.5 mEq, and N, 437 mg; low-potassium-low-protein (deficient) diet, Na and Cl, 3.8 mEq, K, 0.0006 mEq, and N, 1.4 mg; deficient diet supplemented with potassium and protein, Na and Cl, 5.0 mEq, K, 1.9 mEq, and N, 569 mg; deficient diet supplemented with potassium, Na and Cl, 4.1 mEq, K, 1.6 mEq, and N, 1.5 mg; and, deficient diet supplemented with protein, Na and Cl 3.2 mEq, K, 0.0012 mEq, and N, 347 mg. It will be noted that during the fifth week of a 5-week period of consuming a low-potassium and a low-protein diet there was a negative nitrogen balance which, among the several groups of animals, averaged from 39 to 56 mg N per rat per day. Likewise, there was a negative potassium balance in each group of rats, amounting on the average to from 0.05 to 0.09 mEq K per rat per day. The chloride and sodium balances were found to be inconsistent, varying from slightly positive to slightly negative..

When the deficient diet was replaced by the control diet, the rats exhibited a striking gain in weight (an average weight gain of 55 g) and the balances of nitrogen, chloride, sodium and potassium became positive. The average potassium balance of 0.65 mEq per rat per day means that during the 6 days of feeding each rat retained on the average 3.90 mEq of potassium. The largest fraction of this potassium can be accounted for in the skeletal muscle. Further, calculations based on tissue weights revealed that the liver had gained a significant quantity of potassium and the kidneys and heart had gained a small amount. However, such calculations could not account for all of the retained potassium, indicating that it had been stored in other sites as well.

During the 6-day period in which the deficient diet was supplemented with potassium, the rats lost on the average 2 g of body weight. The chloride and sodium balances were found to be negative and the potassium balance was found to be positive in the amount of 0.01 mEq per rat per day. The nitrogen balance continued to be negative, but, on the average, was less negative when potassium was included in the diet than when it was omitted. From Table II, it will be noted that the addition of potassium to the deficient diet led to an increase of the potassium concentration of skeletal muscle and kidney. The major portion of the retained potassium can be accounted for in the skeletal muscle. Calculations based on tissue weights revealed that the liver had gained a small quantity of potassium and only questionable amounts were retained by the kidneys and heart muscle.

Upon supplementing the deficient diet with protein for 6 days the rats exhibited a gain in body weight and registered a positive nitrogen balance. However, the nitrogen retention was not as striking, nor was there such a good growth response (an average weight gain of 17 g), as was observed when the deficient diet was supplemented with both potassium and protein. With protein supplementation, the potassium and protein-deficient rats exhibited negative balances for chloride, sodium and potassium. The total potassium content of the various tissues was calculated from the concentration and the total tissue weight, assuming skeletal muscle is one-third of the body weight, or possibly one-fourth. When protein was added to the deficient diet, there was an insignificant change of total potassium in the heart, a slight increase in the kidney, an appreciable increase in the liver, and a considerable loss from the skeletal muscle. However, the gains of potassium by the liver and kidneys did not account for all of the potassium theoretically lost from the skeletal muscle and not excreted, even when the lower value was assumed for skeletal muscle weight. The increase in total liver potassium was greatest with the supplementation of both potassium and protein, next with the protein supplement, and least with the potassium supplement.

Summary. Balances of nitrogen, chloride, sodium and potassium and changes of the electrolyte content of skeletal muscle, kidney, heart muscle and liver were observed in potassium and protein-deficient rats upon supplementing the diet with (1) potassium and protein, (2) potassium, and (3) protein. Principal attention was focused on the findings encountered when the deficient diet was supplemented with protein, since a redistribution of potassium within the body seemed to occur. Realimentation with protein was attended by an unsatisfactory nitrogen retention and a poor growth response. The plasma potassium concentration became reduced, there was a precipitous fall of the skeletal muscle potassium content, and there were reductions of the potassium concentration of the kidneys, heart muscle and liver. The animals continued to be in a negative potassium balance, but the latter was not of a sufficient magnitude to account for the muscle potassium loss. Calculations based on the tissue potassium concentration and changes in tissue weights indicated a small gain of potassium by the kidneys and a significant gain by the liver. In its redistribution within the body, a fraction of potassium theoretically released from skeletal muscle remained unaccounted for. It was concluded that this potassium entered sites other than the kidneys, heart or the liver.

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Appearance of Soluble and Cross-Reactive Complement-Fixing Antigens on Treatment of Poliovirus with Formalin.* (21808)

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Formalin is currently being used extensively for the inactivation of poliovirus in the production of killed virus vaccines. The antibody response to these products has been studied in humans and monkeys but nothing has been reported to the present time of the effect of formalin on the complement-fixing (CF) antigens of the virus itself. The purpose of this report is to describe certain changes that have been found in the CF activity of tissue culture (TC) grown poliovirus on treatment with formalin in a manner similar to that suggested by Salk(1) for the production of vaccines.

Materials and methods. Virus was grown either in cultures of monkey kidney epithelial cells or, in the case of lot Y-SK II, in HeLa strain human carcinoma cells. Medium 199 (2) was used as the final fluid phase at the time of virus seeding in both types of culture. Large inocula (over 10^6 doses of virus) were used and the fluids harvested 40 hours after inoculation, when most of the cells had degenerated and fallen away from the glass. Those lots with the highest titers were selected for further study. W-S, Brunhilde, and Mexico strains of Type I were studied, and the Y-SK and Leon strains of Types II and III respectively. All except Mexico (kindly supplied by Dr. John F. Enders) had been carried through more than 20 serial TC passages. *Formalinization* was carried out at pH 7 with one part of formalin per 4000 of TC fluid(1). The mixture was incubated at 37°C for 9 days and then dialyzed to remove any free formaldehyde. The product will be referred to here as "vaccine." None of the lots of vaccine produced cytopathic changes in tissue culture. *Fractionation* of both the active virus and vaccine was carried out by centrifugation at 105,000 X G for 2 hours. The upper 4/5 of the supernatant fluid was drawn off and saved. The pellet was resuspended in the remaining fluid and diluted with Medium 199 to the original volume. The former fraction will be referred to as "supernate" and the latter as "sediment." Pooled *sera* from several immunized monkeys were used for Type II (Y-SK strain) and III (Leon strain). The Type I serum was from a single monkey (strain Israel-50) immunized with infected monkey spinal cord. All were heated 20 min at 60°C and used at dilutions between 1/4 and 1/16. *CF tests* were carried out by the modification of the plate test previously described(3). Each antigen dilution was titrated against three concentrations of complement and the difference (avidity) was determined between the amounts of complement fixed in the test and control drops.

Results. Data obtained by titration of 6 different active and formalinized virus preparations are given in Table I. The titrations were carried out using serial 30% increments

TABLE I. CF Titers of Poliovirus Preparations before and after Formalin Treatment.

		Type of antiserum					
		I	II	III	I	II	III
Antigen		Live virus			Formalinized vaccine		
W-S	Wh*	2.1	0	0	2.1	1.2	.9
	Su	0	0	0	2	0	0
	Se	1.7	0	0	4	0	0
Brunhilde	Wh	6.3	0	0	10.7	1.3	0
	Su	1.0	0	0	2.8	1.7	1.0
	Se	8.2	0	0	6.3	1.7	0
Mexico	Wh	2.8	0	0			
	Su	0	0	0	2.2	1.7	0
	Se	4.8	0	0	1.7	0	0
Y-SK M	Wh	0	13.9	0	1.7	3.7	0
	Su	0	0	0	1.7	2.2	0
	Se	>1.7	13.9	0	1.3	3.7	0
Y-SK H	Wh	0	3.7	0	>1.7	<2.2	0
	Su	0	1.7	0	1.0	0	0
	Se	1.7	2.2	0	1.0	<2.2	0
Leon	Wh	0	0	6.3	1.3	1.3	<3.7
	Su	0	0	2.2	2.2	1.3	0
	Se	0	0	3.7	0	0	<2.2

* Wh = Whole; Su = Supernate; Se = Sediment.

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* Aided by a grant from The National Foundation for Infantile Paralysis.

TABLE II. Avidities Found in the CF Titration of Antigen Y-SK M against Antisera of Each Virus Type.

Antigen dilution	Live virus preparation									Formalinized vaccine preparation								
	Whole			Supernate			Sediment			Whole			Supernate			Sediment		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Undil.	–4		–4	–4	–5	–4	*1*		–5	*2*		–4	*1*	*1*	–5	*4*		–5
1.3	–4		–4	–4	–5	–5	*1*		–5	*4*		–4	*2*		–5	*1*		–4
1.7	–2		–4	–4	–4	–4	*3*		–2	*4*		–1	*0*	*2*	–2	–2		–4
2.2					–5					–1		–5	–3	*2*	–5	–4	*5*	–5
2.8					–5					*0*		–5	–2	–3	–5		*4*	
3.7		*6*			–5			*6*		–2	*3*	–5					*1*	
4.8		*6*			–5			*4*			–1			–5			–3	
6.3		*5*						*4*			–2						–3	
8.0		*3*						*2*			–3			–5			–4	
10.7		*2*						*1*			–3						–4	
13.9		*3*						*3*			–4			–5				
18.0		–3						–4										

Positive values are in italic type. Titers are indicated by a horizontal line.

in antigen dilution, *i.e.*, undiluted, 1/1.3, 1/1.7, etc. While the limits of reproducibility of the method were greater than the 30% increment, the large number of different dilutions tested acted as checks one on the other. The values given for the whole W-S virus and vaccine represent the mean of 4 and 7 titrations respectively. The standard deviations for these titrations ranged from ± 0.57 to ± 0.77 with an average of ± 0.68. Except for the W-S series, all titrations were carried out with one dilution of each preparation per test plate. Thus errors, such as partial drying, which might affect one plate more than another, affected all the preparations on the plate similarly and showed up only as a greater or lesser avidity than those found for the bracketing dilutions. The avidities that were found for one lot are given in Table II as an example of the reproducibility of the method on which the titers are based. The antigen titers were taken as the highest dilution yielding a positive avidity (and not preceded by negative avidities of greater numerical value).

With the exception of the Type I cross in the Type II sediments, the data of Table I show that all the live virus preparations gave type specific reactions. The supernates of the virus preparations contained only a small part of the activity of the unfractionated material, whereas the titers of the sediments were not significantly different from those of the whole.

Formalinization caused a variable loss of homotypic titer. In some cases this was insignificant, but in others (Y-SK M and Leon) more than 50% of the initial titer was lost. In every case there was an increase in the CF activity remaining in the supernate. In every case also there was more heterotypic cross reaction than in the corresponding live virus preparation. The reciprocal cross between Types I and II vaccines were found more consistently than the crosses between these antigens and Type III serum. However, the single specimen of Type III vaccine that was studied crossed with both I and II sera. Cross reactions appeared in both the supernate and sediment fractions, though in some preparations they were stronger in the supernate.[†] Heat-inactivated (56°C for 30 minutes) control preparations did not produce cross reactions, in confirmation of Svedmyr *et al.*(5).

Discussion. There are at least 2 possible explanations of the increase found, after formalin treatment, in soluble (non-sedimentable) antigen and in cross reactions between poliovirus types. The formalin may have reacted with some non-sedimentable component of the preparation and converted it to a form capable of giving a CF reaction, or the formalin may have caused the breakup of a larger particulate component, exposing a group antigen which had previously been masked. In either case the component involved was associated with the virus cultivated in both monkey kidney and HeLa strain cultures. Of the two possibilities, the breakup of a particle seems the more probable, as there is corroborative evidence of common antigens in different poliovirus types. Thus cross CF reactions occur frequently among newly isolated strains of poliovirus(4), monovalent formalinized vaccine may stimulate the production of heterotypic CF antibodies(3), simultaneous increases in more than one type of CF antibody are frequent in natural infections except in the very young exhibiting primary poliomyelitic infection(5), and a sensitization to Type I or III vaccines may be afforded by previous experience with Type II (and *vice versa*)(6). The particulate component affected by the formalin might be either the infective virus or the smaller 12 mμ component present in infected tissue cultures, recently referred to by Schwerdt(7) and Taylor(8), as both would have been sedimented under the conditions of our experiments.

[†] Not all strains treated with formalin behave in this fashion. Three samples of commercial monovalent vaccine gave monotypic, although weak, CF reactions. The vaccines were made from Mahoney, MEFl, and Saukett strains.

Summary. Poliovirus in tissue culture fluid was treated with dilute formaldehyde and fractionated by ultracentrifugation. Most of the complement-fixing antigen in the active virus preparations could be sedimented at 105,000 x gravity for 2 hours. However, in the formalin-treated samples a soluble antigen appeared which remained in the supernatant fluid. The active virus preparations used were almost completely type specific in their complement-fixing reactions, but after treatment with formaldehyde, cross reactions were found between all types.

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A Histochemical Study of Normal and Cirrhotic Livers.* (21809)

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The purpose of this paper is to present a histochemical characterization of the normal liver cell and to compare this characterization with one obtained by the application of the same procedures to liver cells in cirrhotic livers.

Materials and methods. Liver tissue for this study, obtained at the time of autopsy and preserved in the deep freeze, was studied by the histochemical procedures in question as described by Pearse(1) or as described in the reference given. Three histopathologic patterns were noted with the hematoxylin and eosin stain. In one group the microscopic picture in the liver was one of a moderate increment in periportal fibrous tissue, and infiltration of chronic inflammatory cells and proliferation of bile ducts. The intervening parenchyma was divided into varying sized lobules by the fibrous tissue, but the liver cells appeared normal. There were 3 cases in this group. In a second group the livers displayed a marked increase in proliferating periportal fibrous tissue accompanied by a generous inflammatory cell exudate and considerable bile

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duct proliferation. The majority of the small bile canaliculi scattered throughout the greatly reduced parenchyma were plugged with bile. The parenchyma was broken into nodules by the fibrous tissue but the liver cells appeared normal. There were 3 cases in this group, diagnosed as follows: post hepatitic cirrhosis, congenital atresia of the bile ducts with biliary cirrhosis, and Lannec's cirrhosis. In the third group the histologic picture was one of a moderate degree of periportal fibrous tissue proliferation which broke the parenchyma into nodules with a moderate infiltration of chronic inflammatory cells into the fibrous tissue. The hepatic cells displayed a marked degree of fatty metamorphosis. Virtually the entire cytoplasm of the majority of the liver cells was distended by fatty vacuoles which on occasions coalesced to form small fatty cysts. There were 2 cases in this group. For comparison 2 livers were selected for study. One was obtained from a patient dying acutely from trauma and this liver was considered normal. The other was obtained from a patient with complete extrahepatic block due to metastatic cancer in the porta hepatis. This liver showed acute ascending cholangitis with bile stasis but with no evidence of cirrhosis or fatty metamorphosis.

Results. All the liver cells contain the following lipid components; triglycerides, fatty acids, phospholipids, cholesterol and its esters, acetal lipids, phosphatides, and triolein and oleic acids. In the non-fatty liver cells these compounds are present as fine particles. In the fatty liver cells they are present as large globules which distend the cytoplasm and which are predominantly triglycerides as demonstrated by a red color when stained with Nile blue sulfate. In the normal liver all the lipid takes a blue stain with Nile blue sulfate indicating the presence of acidic lipids.

All of the liver cells contain potassium, glycogen, free aldehyde groups, sulfuric acid esters of mucopolysaccharide origin, protein bound sulfhydryl groups(2), mucoproteins and calcium. These substances were evenly distributed in the cytoplasm of the non-fatty liver cells. In the fatty cells these substances were compressed to the periphery of the cell by the fatty vacuole.

Non-specific esterase(3) was finely distributed throughout the cytoplasm of the non-fatty cells. In the fatty cells the esterase activity was represented by large clumps and appeared increased in amount.

RNA, as demonstrated by a loss of cytoplasmic basophilia after ribonuclease digestion, was present in generous amounts in the cytoplasm of the normal liver cells, and in the cytoplasm of the liver cells from the cirrhotic patients who were not suffering from severe liver disease.

In the 2 patients suffering from clinically active liver disease, *i.e.* post hepatitic cirrhosis, and congenital atresia of the bile ducts, the cytoplasmic RNA was markedly reduced and compressed to the periphery of the cell.

Alkaline phosphatase was present in a few of the Kupffer cells in all of the livers studied. In the patients with clinical jaundice at the time of post-mortem, due to extra-hepatic obstruction or cirrhosis; alkaline phosphatase was also present in the bile and in parenchymal cells by the central vein.

Vit. C was present in random small clusters of liver cells in the fatty livers, and in rare cells in the livers with active inflammation and fibrous tissue proliferation, but absent in the livers with minimal cirrhosis and the 2 control livers.

The liver cells did not give a reaction for or stain for arginine, mucin, peroxidase, acid phosphatase, tyrosine, or free carbonyl groups (4).

The fibrous tissue stroma give slightly positive reactions or stains for lipids, potassium, calcium, mucoproteins, mucopolysaccharides, protein bound sulfhydryl groups, indole and non-specific esterase. It gave a moderately intense reaction for amyloid and free aldehyde groups.

Discussion. Alkaline phosphatase has been reported to be increased in experimentally induced cirrhosis in mice(7) and in active human cirrhosis(8). These studies do not state whether or not the mice or the humans displayed clinical jaundice at the time of the study. In view of the results reported here and elsewhere it seems likely that histochemically demonstrable alkaline phosphatase is not evident in significant amounts in the liver unless the subject has clinical jaundice.

Esterase activity has been reported as slightly decreased in experimentally induced cirrhosis(7). In these animals there was slight fatty metamorphosis. In the patients studied in this report esterase activity was essentially normal until marked fatty metamorphosis appeared and then esterase activity increased.

In the experimental cirrhosis of mice reported by Stowall, *et al.*(7) liver lipid was decreased. In this study liver lipid was normal until fatty metamorphosis was evident, and at this time the lipid changed its physical state and staining characteristics.

The cytoplasmic basophilia and its loss with ribonuclease digestion reported in these livers are in agreement with similar findings by Szanto and Popper(6). They demonstrate that the RNA content of the liver cell is normal in obstructive jaundice, and inactive cirrhosis; but that it is greatly depleted in active cirrhosis with liver cell damage, and in the liver cells distended with fat. This latter observation is in accord with the findings of Eckhardt, *et al.*(5).

Summary. 1. Liver cell contains lipids, potassium, free aldehyde groups, sulfuric acid esters of mucopolysaccharide origin, protein bound sulfhydryl groups, mucoproteins, calcium, non-specific esterase, ribosenucleic acid and vit. C. 2. The lipid of the liver cell showing fatty metamorphosis is histochemically different than normal liver cell lipid. 3. The non-specific esterase activity is increased and clumped in the fatty liver. 4. Cytoplasmic liver cell RNA appears to be decreased in patients with morphologically active cirrhosis. 5. In the presence of clinical jaundice, alkaline phosphatase activity is increased in the Kupffer cells, in the liver cells adjacent to the central veins in the bile plugging the ducts. 6. Fatty liver appears to contain more vit. C than the normal liver.

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An Improved Urinary Excretion Test as an Assay for Intrinsic Factor.* (21810)

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Progress toward the isolation and purification of intrinsic factor has been hampered by the lack of a rapid and readily available assay method. The usual method necessitates the use of pernicious anemia patients in relapse (2). Relapsed patients, suitable for assay of intrinsic factor preparations, are so rare that more rapid methods for the determination of intrinsic factor using pernicious anemia patients in remission have been proposed. Heinle *et al.*(3) reported that pernicious anemia patients in remission excreted in the feces almost all of an oral dose of vit. B_{12} labeled with Co^{60} (vit. $B_{12}Co^{60}$). The simultaneous oral administration of intrinsic factor and vit. $B_{12}Co^{60}$ significantly reduced the fecal excretion because the intrinsic factor enabled some of the vit. B_{12} to be absorbed. Another approach to measuring the uptake of orally administered vit. B_{12} in patients with pernicious anemia was made by Glass *et al.* (4). Their technic involves the measurement of hepatic uptake of vit. $B_{12}Co^{60}$ by scanning the liver with a scintillation detector. Schilling(5) administered an oral dose of 2 μg of vit. $B_{12}Co^{60}$ together with intrinsic factor to pernicious anemia patients in remission and collected their urine for the next 24 hours. No radioactivity was excreted in the urine unless a large "flushing" dose of unlabeled vit. B_{12} was injected and a source of intrinsic factor was given orally. It may be concluded that intrinsic factor acts to enable vit. B_{12} to enter the tissues of such patients and the "flushing" injection serves to detect this absorption.

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* A preliminary report was presented at the Eastern Section of the Am. Fed. of Clin. Research, Washington, D. C., Dec. 1954.(1)

We have investigated certain aspects of the urinary excretion test (U.E.T.) with a view to improving it in order to obtain more quantitative information. The present paper describes some of these investigations as well as the modifications and precautions necessary in the use of the U.E.T. as an assay method.

Materials and methods. Patients with typical Addisonian pernicious anemia in remission were from the wards and outpatient department of Kings County Hospital, Brooklyn, N. Y. They ranged in age from 35 to 84. The control group, mostly laboratory workers, were healthy adult subjects with normal hematological values. They ranged in age from 24 to 70. Two μg of vit. $B_{12}Co^{60}$ were given orally either alone or together with an intrinsic factor preparation. The specific activity of the radioactive vit. B_{12} was 0.25 μc/μg. No food was given in the morning and for 2 hours after the oral dose. One to 4 mg of unlabeled vit. B_{12} was injected intramuscularly immediately after the oral dose of vit. $B_{12}Co^{60}$ was given. Twenty-four hour urine collections were obtained and the radioactivity of the specimens was counted in a 4 ml well-type scintillation counter. Enough counts were taken to reduce the standard error to below 5%. When necessary the urine was first concentrated 10 to 20-fold by evaporation. Background determinations were obtained twice daily, usually before and after a series of counts. A Co^{60} reference standard was used to check any day-to-day variation in the instrument.

TABLE I. Urinary Excretion of Vit. $B_{12}Co^{60}$ following Oral Administration of 2 μg Vit. $B_{12}Co^{60}$ and Daily Intramuscular Injections of 1 to 4 mg Unlabeled Vit. B_{12} in 22 Normal Subjects.

	% excretion		
Patient	1st day	2nd day	3rd day
1 mg unlabeled vit. B_{12} inj. daily			
A. A.	6.4		
J. A.	9.5	5.2	
W. B.	9.9		
J. E.	12.3		
J. E.	15.9	6.7*	1.6*
H. F.	16.3		
P. F.	11.0		
J. G.	9.5		
R. K.	9.1	3.7	
A. P.	9.2		
A. R.	5.8		
G. S.	17.0		
A. S.	7.3		
I. S.	14.7		
S. T.	5.4		
W. W.	16.5		
W. W.	10.5	8.6*	3.0*
2 mg unlabeled vit. B_{12} inj. daily			
L. H.	9.2	1.6	1.2
4 mg unlabeled vit. B_{12} inj. daily			
A. A.	9.2	3.4	
L. E.	11.1	2.2*	.2*
F. S.	29.2	5.0	
G. S.	12.6	2.9	
Avg	11.7	4.4	1.5

* 2 mg unlabeled vit. B_{12} injected.

Results. Effect of Consecutive Flushing Injections. As can be seen from Table I, normal subjects excreted definite and significant amounts of radioactivity on the second and third day following second and third intramuscular injections of unlabeled vit. B_{12}. Pernicious anemia patients given vit. $B_{12}Co^{60}$ by mouth without intrinsic factor absorbed very little of the oral dose and hence intramuscular injections of vit. B_{12} on the second day did not flush out significant amounts of radioactivity in the urine (Table II). Larger flushing injections of 2 mg and 4 mg of vit. B_{12} failed to increase the rate and amount of excretion of vitamin $B_{12}Co^{60}$ in the urine (Tables I and II).

TABLE II. Urinary Excretion of Vit. $B_{12}Co^{60}$ following Oral Administration of 2 μg Vit. $B_{12}Co^{60}$ and Daily Intramuscular Injection of 1 to 4 mg of Unlabeled Vit. B_{12} in 35 Pernicious Anemia Patients.

	% excretion				
	Without intrinsic factor		With intrinsic factor Reference sample		
Patient	1st day	2nd day	1st day	2nd day	3rd day
	1 mg unlabeled vit. B_{12} inj. daily				
J. A.	.3		7.8		
A. B.	2.6				
E. B.	.0				
H. B.	.0				
A. C.	.4	.0	3.3		
B. C.	.5	.1			
D. C.	2.3	.7	4.3	3.9	
R. E.	.2		11.6		
R. F.	.4	.0	6.7		
B. G.	.1	.0	8.5	4.4	
D. G.	.4	.0	2.2	1.2	
S. G.	§	.0	5.4	2.1	
T. G.	.6‡		2.7	1.4	
A. K.	.0	.0	3.2	2.4	
M. K.	.1		12.7		
N. L.	.5	.0	3.7	.5	
F. M.	.3		6.2		
F. P.	.2	.3	1.8	.0	
A. R.	.3	1.0	3.1	2.4	
S. R.	.2		2.8	2.7	
S. R.	.1		6.1		
A. S.	2.4		12.5		
F. S.	1.5‡		6.4	3.3	
G. S.	.5	.1	3.3	1.7	
L. S.	1.3	.3	10.1	5.2	
M. S.	.0	.3	6.2	2.2	
S. S.	.6‡		7.6	4.5	
S. W.	1.0‡		5.6	2.2	
G. W.	.2	.2	1.7	2.2*	.9*
M. W.	.8		16.4		
	4 mg unlabeled vit. B_{12} inj. daily				
E. B.	.1		2.8	1.8	
F. G.	.5	.0	13.8	5.4	
M. J.	.6	.5	2.3	3.4	1.1
E. K.	2.7			8.0†	
J. S.	1.0	.0	2.4	1.5	.6
Avg	.7	.2	6.1	2.6	.9

* 2 mg unlabeled vit. B_{12} injected.

† Value for total 48 hr collection. Not included in 1st or 2nd day averages.

‡ Tested at conclusion of 2nd day with the intrinsic factor reference sample; therefore, value might be slightly high.

§ Urine accidentally discarded.

An intrinsic factor concentrate, #186-1, similar to the ammonium sulfate fraction described by Williams *et al.*(6), fully effective in the treatment of pernicious anemia patients in relapse at a dosage of 25 mg per day was used† to test the effect of intrinsic factor in the U.E.T. in pernicious anemia patients. Fifty mg of this preparation increased the excretion value into the range obtained with normal persons (Table II). Inasmuch as pernicious anemia patients upon receiving intrinsic factor were then able to absorb vit. B_{12}, flushing injections on the second and third day resulted in excretion of additional radioactivity on both days.

Use of a Reference Intrinsic Factor Sample. Due to the considerable variation of excretion values from patient to patient it was considered advisable to study the use of an intrinsic factor preparation of known potency to facilitate quantitative evaluation of other intrinsic factor preparations. The use of this procedure is shown in Table III. With the first patient, F. G., the excretion of 4.6% was in the usual range of response of pernicious anemia patients to intrinsic factor preparations (1.8 to 19.2%—Table II); however, since this patient gave an excretion value of 19.2% with the reference sample it is apparent that sample 230 at the dosage employed gave a submaximal response. At the same dosage this preparation also gave a submaximal response in a clinical test. By comparison, patient N.L. gave an excretion value of 4.0% indicating a maximum response to sample 235 since the

TABLE III. Use of a Reference Sample of Intrinsic Factor for Quantitative Evaluation of Intrinsic Factor Activity.

Day	$B_{12}Co^{60}$, μg	Intrinsic factor concentrate	Excretion in urine, %
Patient F. G.			
1	2	0	.5
2	0	0	.0
3	2	Unknown #230	3.3
4	0	0	1.3
5	0	0	.0
6	2	Reference sample	13.8
7	0	0	5.4
Patient N. L.			
1	2	0	.5
2	0	0	.0
3	2	Unknown #235	3.1
4	0	0	.9
5	2	Reference sample	3.7
6	2	0	.6

F. G. was injected daily with 4 mg unlabeled vit. B_{12} and N. L. with 1 mg.

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† We are indebted to Dr. Tom D. Spies, Nutrition Clinic, Hillman Hospital, Birmingham, Ala., for clinical evaluation of the intrinsic factor preparations.

TABLE IV. Effect of Multiple Injections of Vit. B_{12} on Urinary Excretion of Orally Administered Vit. $B_{12}Co^{60}$ in Normal Healthy Males.

Day	Vit. $B_{12}Co^{60}$, µg	Inj. vit. B_{12} mg	Excretion, %
Patient P. F.			
1	2	1	11.0
2 to 6	0	4 (each day)	*
7	2	4	2.3
Patient A. P.			
1	2	1	9.2
2 to 6	0	4 (each day)	*
7	2	4	.2

* Urine was not collected.

reference sample gave a similar excretion value of 4.3%.

Effect of Large Doses of Parenteral Vit. B_{12}. The effect of large and repeated daily injections of unlabeled vit. B_{12} on the 24-hour excretion of a test dose of vit. $B_{12}Co^{60}$ was investigated in 2 normal individuals (Table IV). It is evident that the injection of 25 mg of unlabeled vit. B_{12} over a period of 7 days resulted in a significantly decreased excretion of radioactivity. This was not apparent with pernicious anemia patients receiving similar amounts of parenteral vit. B_{12} (last 5 patients in Table II), however, a decrease was observed under somewhat different conditions by Baker and Mollin(7) and Callender and Evans(8) with pernicious anemia patients.

Effect of large doses of intrinsic factor. Baker and Mollin(7), using a fecal excretion technic, have reported that increasing amounts of intrinsic factor proportionally increased the absorption of labeled vit. B_{12}. On the other hand, Glass *et al.*(9) have reported that doubling of the dose of certain intrinsic factor preparations which gave maximum hepatic uptake resulted in decreased uptake of vit. $B_{12}Co^{60}$ in several individuals. To investigate this further each of 2 pernicious anemia patients were given 50 mg and 500 mg of the intrinsic factor reference sample (Table II). The vit. B_{12} content of this batch of intrinsic factor concentrate was 0.1 µg/g. As can be seen from Table V considerably more radioactivity was excreted in the urine with the larger dose of intrinsic factor. After receiving 50 mg of intrinsic factor, patient G.W. excreted more radioactivity on the second day than the first. With 500 mg the greatest excretion occurred on the third day. These are the first instances of the maximum excretion taking place after the first day.

Discussion. In the present method, flushing doses of unlabeled vit. B_{12} are given consecutively on the 1st, 2nd and 3rd days following the administration of vit. $B_{12}Co^{60}$ with intrinsic factor. This procedure makes it possible to use the same patient for a series of assays of intrinsic factor. Earlier procedures did not enable this to be carried out, for failure to consider the fact that more than one flushing dose is needed to cause the maximal amount of radioactivity to be excreted leads to serious error in the assay of two intrinsic factor preparations with one patient. For example, if a highly active preparation was tested on one day followed by a totally inactive preparation on the second day, the radioactivity excreted on the second day could amount to 50% of that excreted on the first day in spite of the inactivity of the second preparation. The use of multiple flushing doses permits the use of a reference sample of intrinsic factor in each test of an unknown preparation. The excretion value of the unknown preparation must always be compared with the value obtained by the reference sample. The use of multiple flushing injections

TABLE V. Effect of Large Amounts of Intrinsic Factor on Urinary Excretion of Vit. $B_{12}Co^{60}$ in Pernicious Anemia Patients.*

Day	Vit. $B_{12}Co^{60}$, µg	Intrinsic factor Reference sample, mg	Excretion in urine, %
Patient E. B.			
1	2	0	.1
2	2	50	2.8
3	0	0	1.8
4	2	500	4.1
5	0	0	3.0
Patient G. W.			
1	2	0	.2
2	2	50	1.7
3	0	0	2.2
4	0	0	.9
5	2	500	3.1
6	0	0	4.3
7	0	0	5.7
8	0	0	.7

* Both patients injected daily with 4 mg of vit. B_{12}.

to increase the amount of the absorbed radioactive vit. B_{12} which is excreted in the urine accentuates the difference in the excretion values between normal individuals and pernicious anemia patients not receiving intrinsic factor and has the additional advantage of improving the removal of radioactivity from the tissues of the subject. Furthermore, by this procedure a delayed excretion of radioactivity will be revealed in individuals such as G.W. (Table V). Since Callender and Evans(8) and MacLean and Block(10) have shown that on the average about one-third of the vit. B_{12} absorbed from the gut is excreted in the urine in 24 hours using one flushing dose, it is apparent from the values in Table II that use of 3 flushing doses on three consecutive days would flush out approximately half the vit. $B_{12}Co^{60}$ absorbed.

There are two possible explanations for the inhibitory effect of large parenteral doses of unlabeled vit. B_{12} on the absorption of the vit. $B_{12}Co^{60}$ from the gut: (1) there may exist a regulatory mechanism in the gut wall under the influence of the vit. B_{12} tissue levels; (2) high tissue levels result in re-excretion of unlabeled vit. B_{12} in the gut thus diluting the vit. $B_{12}Co^{60}$ so that less radioactivity is absorbed. The actual amount of vit. B_{12} absorbed may be little changed. The second explanation seems to be the most likely; Callender and Evans(8) and Baker and Mollin(7) have also shown that increased tissue levels of unlabeled vit. B_{12} result in decreased absorption of radioactive vit. B_{12}.

Our results showing that an increase in the intrinsic factor dosage leads to an increased excretion of radioactivity in the urine are in agreement with the observation of Baker and Mollin that increasing dosage of intrinsic factor leads to increased absorption of radioactive vit. B_{12}. The decrease in absorption observed by Glass *et al.*(9) with large doses of intrinsic factor may be attributed to the unlabeled vit. B_{12} content of the crude preparations employed which would dilute the radioactive vit. B_{12}. The intrinsic factor preparation used in our experiments contained a negligible quantity of vit. B_{12}. Indeed, Toporek *et al.*(11) have shown that certain clinically potent intrinsic factor preparations containing significant amounts of vit. B_{12} appear to have low activity as estimated by the U.E.T. The average 24-hour excretion values of 11.7% for normal subjects and 6.1% for pernicious anemia patients receiving intrinsic factor obtained in the present study are in good agreement with the corresponding average values of 14.7% and 9.5% obtained by Schilling(12) who used a single flushing dose. Such comparisons should take into consideration the dosage of radioactive vit. B_{12} employed. A value of 25% for normal subjects was obtained by MacLean and Block(10) who used 0.7 μg of vit. $B_{12}Co^{60}$ as the oral dose.

To make the urinary excretion technic more readily adaptable to clinic and outpatient departments of hospitals, it has been found satisfactory to inject the 1 mg flushing dose of unlabeled vit. B_{12} simultaneously with the oral dose of vit. $B_{12}Co^{60}$.

Our findings lead to the following proposed procedure for the urinary excretion test in the assay of intrinsic factor preparations with pernicious anemia patients in remission. *Day* 1—Two μg of vit. $B_{12}Co^{60}$ (0.5 μc) are given orally and 1 mg of unlabeled vit. B_{12} is simultaneously injected intramuscularly. Urine is then collected for 24 hours each day and examined for radioactivity. *Day* 2—One mg of unlabeled vit. B_{12} is injected intramuscularly. *Day* 3—Two μg of vit. $B_{12}Co^{60}$ together with the intrinsic factor preparation of unknown potency are given orally. One mg of unlabeled vit. B_{12} is injected intramuscularly. *Day* 4—One mg of unlabeled vit. B_{12} is injected intramuscularly. *Day* 5—Two μg of vit. $B_{12}Co^{60}$ and the intrinsic factor reference sample are given orally. One mg of vit. B_{12} is injected intramuscularly. *Day* 6—One mg of vit. B_{12} is injected intramuscularly.

Summary. A procedure is suggested for the assay of preparations of intrinsic factor by measuring the urinary excretion of radioactivity following dosage with vit. B_{12} labeled with cobalt 60. It was found important to use more than one "flushing" dose of unlabeled vit. B_{12} to produce maximum excretion of radioactivity. By use of this precaution, the same patient could be used for assaying several preparations of intrinsic factor in succession. The injection of large amounts of un-

labeled vit. B_{12} appeared to decrease the uptake of small doses of labeled vit. B_{12} when subsequently administered orally. There appeared to be some proportionality between the dosage of intrinsic factor and the amount of radioactivity excreted in the test.

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Propagation in a Fluid Medium of a Human Epidermoid Carcinoma, Strain KB. (21811)

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The specific amino acid and vitamin requirements of a mouse fibroblast (strain L) and of the human carcinoma cell (strain HeLa) have recently been identified(1-4). It has been shown that these cells can be grown in a medium consisting of 13 essential amino acids, 7 essential vitamins, glucose, salts, and serum protein. For stock cultures the essential factors, each in the concentration necessary for maximal growth, were supplemented with 10% fresh human serum. The HeLa cell has now been serially propagated in this medium for 8 months, with an average generation time during the logarithmic phase of growth of approximately 48 hours.

It seemed possible that the optimal medium as defined for the HeLa cell (Table I) might permit the direct isolation of new lines of human cells. The present paper describes the successful isolation and propagation, directly on a glass surface, and without intervening cultivation in a plasma clot, of an epidermoid carcinoma of the floor of the mouth (strain KB).

Experimental. A 54-year-old white man (KB) developed an ulcerated friable tumor involving the floor of the mouth and the tongue 5 weeks after the extraction of 9 teeth on Oct. 29, 1954. On Dec. 21, 1954, sections of biopsy material indicated the presence of a poorly differentiated epidermoid carcinoma. The microscopic appearance of the tumor in stained section is shown in Fig. 1. The courtesy of Dr. Louis B. Thomas in supplying both the section and his descriptive notes is greatly appreciated.

Some of the biopsy material from the tu-

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* The author acknowledges gratefully the technical assistance of Clara Horton.

† Public Health Service, U. S. Department of Health, Education, and Welfare.

TABLE I. Medium* Used for Isolation and Cultivation of a Human Epithelioid Carcinoma (Strain KB).

L-Amino acids (mM)		Vitamins (g/ml)		Salts (%)	
Arginine	.1	Biotin	10^{-6}	NaCl	.68
Cystine	.05	Choline	”	KCl	.04
Glutamine	1-2	Folic acid	”	$NaH_2PO_4 \cdot H_2O$	.014
Histidine	.05	Nicotinamide	”	$NaHCO_3$	.22
Isoleucine	.2	Pantothenic acid	”	$CaCl_2$	.02
Leucine	.2	Pyridoxal	”	$MgCl_2$	.008
Lysine	.2	Thiamin	”		
Methionine	.05	Riboflavin	10^{-7}	Misc.	
Phenylalanine	.1			Glucose	.1%
Threonine	.2	Serum protein		Penicillin	.005%
Tryptophan	.02			Streptomycin	.005%
Tyrosine	.1			Phenol red	.0005%
Valine	.2				

* Factors here listed were those previously shown to be essential for growth of HeLa cell (2,3), and concentrations of the amino acids are approximately twice those found optimal for that cell.

mor mass was made available for tissue culture through the courtesy of Dr. Robert Smith. The tissue was finely minced with scissors and suspended in approximately 25 ml of the HeLa growth medium. Difco trypsin "1:250" was added to a final concentration of 0.25%, and the suspension placed on a "Magnamix" stirrer for the separation of discrete cells, as described by Youngner(5) for the preparation of dispersed suspensions of monkey kidney epithelium. After 10 minutes, the turbid supernatant fluid was withdrawn and centrifuged at 400 rpm for 5 minutes. The sediment of discrete cells, cell clumps and debris was resuspended in approximately 15 ml of fresh fluid, without washing. Two ml of this suspension were then placed directly in a number of culture flasks with a surface area of approximately 15 sq. cm.

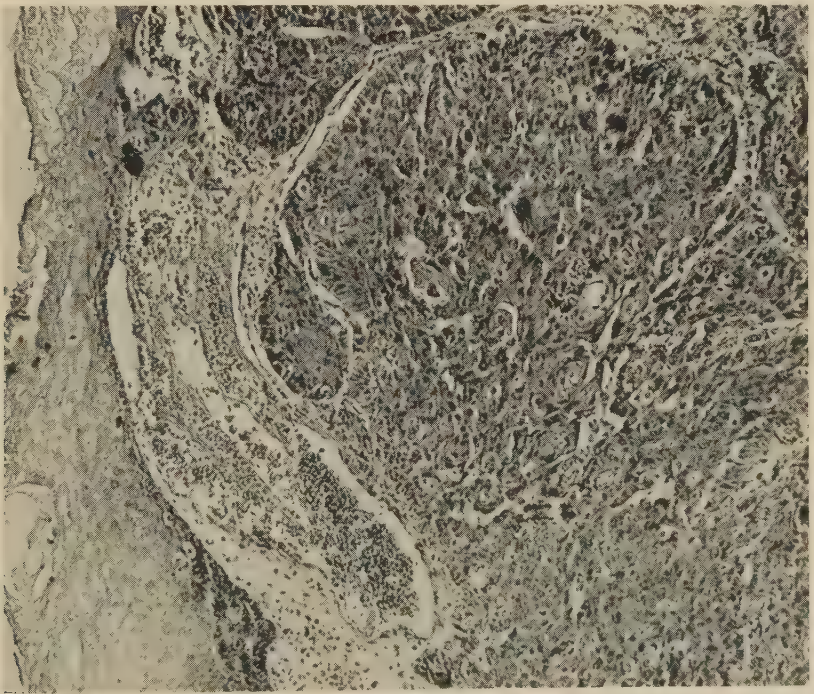

FIG. 1. Microscopic appearance of the KB tumor. H & E stained section, 105×.

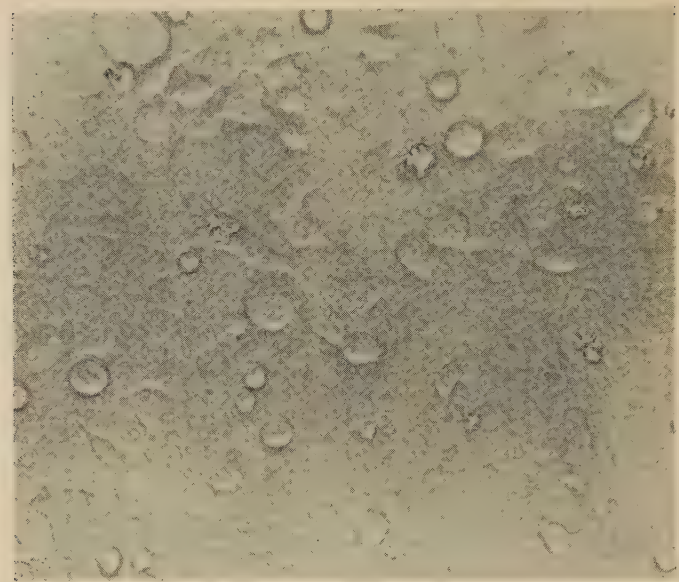

FIG. 2. A 2-day culture of the KB cell in medium of Table I, 180×.

Many of the cells had adhered to the glass in 24 hours. The culture fluid was changed on the second day and every 2 days thereafter, without disturbing those adherent cells. Growth was at first extremely slow. On Jan. 3, 1955, 13 days after the initial planting, the culture was divided for the first time. The cell layer in 3 of the flasks was removed from the glass and separated into small clumps by the addition of warm culture medium containing 0.25% commercial trypsin. The dispersed cells were pooled, sedimented at 400 rpm, resuspended in fresh medium, and now placed in 2 larger flasks, each with a surface area of approximately 30 sq. cm. The rate of growth was now much faster. A week later, the cells of one flask were transferred to a one-liter Blake bottle, with a surface area of 150 sq. cm. By this time the line was well established. The rate of growth had stabilized, with a generation time in the logarithmic phase of growth of approximately 30 hours. The rate of growth has since remained at this high level.

TABLE II. Rate of Growth of Strain KB in HeLa Medium of Table I, Supplemented with 10% Whole Human Serum.

Inoculum × 10^4	Cell count in day No. 1	2	3	4	5
22.5	—	104	282	447	382

Fig. 2 shows the microscopic appearance of a young culture of the cell. The rate of growth in a typical experiment is shown in Table II.

The susceptibility of this new line of human tumor cells to a number of viruses is under present study, as are its specific growth requirements.

Summary. An epidermoid carcinoma of the floor of the mouth was cultivated directly onto a glass surface in a medium consisting of 13 amino acids, 7 vitamins, glucose, salts, and 10% human serum. All of the defined components of this medium had previously been shown to be essential for the propagation of the HeLa cell, and all were used at concentrations permitting maximal growth of that cell. The new line (KB) has been grown continuously in this medium for 5 months, and maintains a generation time in the logarithmic phase of growth of approximately 30 hours.

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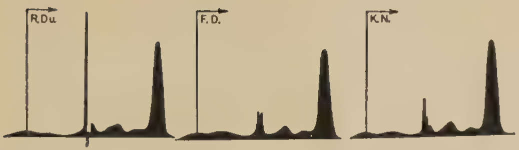

FIG. 1. Electrophoretic patterns of serum of 3 phenylketonuric individuals as determined after 250 min. migration at pH 8.4-8.6. Patterns are essentially normal except for characteristic anomalies associated with the β-globulins.

should be noted, however, that these anomalies were not found in all individuals, nor were they found on all occasions in the serum of the same individual. Only 8 of the 17 patients studied showed these abnormal patterns. It may be significant that all but 3 of the younger children (those below 8 years of age) showed these anomalies at one time or another. It should be reemphasized that all of the serums examined by us were from fasting individuals.

Some of the younger individuals were maintained on diets low in phenylalanine(5), and it was possible to obtain a series of runs at different levels of blood phenylalanine from these. An example is shown in the series of 4 patterns taken on the serum of the same individual (J.Du.) and illustrated in Fig. 2. The first serum, taken before any dietary change, shows the β-lipoprotein anomalies, which disappeared after prolonged maintenance at a normal level of blood phenylalanine. The final serum shows the recurrence of the abnormal pattern after the child had been returned to a natural diet, which resulted in an elevation in the blood level of phenylalanine to the previous high level. In addition to J.Du., whose serum patterns are shown in Fig. 2, A.Du., F.D., R.Du., and M.J. have been maintained on synthetic diets low in phenylalanine for periods of 3 to 9 months. In each case the level of serum protein remained normal and the distribution of serum protein was essentially unchanged, except for small increases in γ-globulin in some cases. These data provide evidence that these diets are adequate for normal synthesis of serum proteins and, as shown elsewhere(5), for growth, despite the restricted intake of phenylalanine.

There have been few earlier studies of serum proteins in phenylketonuria. Shedlovsky and Scudder(14) reported a single observation on a 38-year-old individual with an apparently normal distribution of plasma proteins. Kondritzer(15) found, in agreement with the present studies, a slightly higher globulin level as determined by a method of salting-out the proteins.

Discussion. The most important aspect of any new biochemical abnormalities observed in phenylketonurics is a possible relationship to the development of the mental defect. The finding that abnormal β-globulins are occasionally observed is of interest because this fraction contains lipoproteins, and individuals with other types of mental diseases, such as Schüller-Christian's disease, Gaucher's disease, Nieman-Pick's disease, and Tay-Sach's disease manifest an abnormal accumulation of lipides in cells of the central nervous system, although increased serum lipides may not be present(16). This type of pathology, however, has not been present in phenylketonurics who have been brought to autopsy(17). The presence of abnormal β-lipoproteins is not correlated with the level of serum phenylalanine or the degree of mental impairment; this is indicated by the cases reported here. J.Du., the patient whose serum patterns are shown in Fig. 2, had one of the most distinctive abnormal patterns observed during this study,

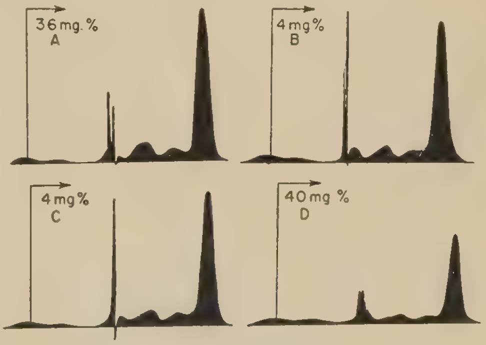

FIG. 2. Electrophoretic patterns of serum of J. Du. as determined at different times. Amount of serum phenylalanine is given with each pattern. Run *A* was made when the child was being fed a normal diet; *B*, after maintenance for 6 wk on a diet low in phenylalanine; *C*, after maintenance on the low phenylalanine diet for 3 mo; *D* was run a year after returning to a natural diet with the accompanying elevated serum level of the amino acid.

yet at the time she was first observed she appeared to be normal in physical and mental development. On the other hand, F.D. with a similar pattern was very retarded, with an I.Q. of approximately 20. Both C.W. (I.Q. 70) and D.D. (I.Q. 22) showed essentially normal patterns, despite the mental differences.

In contrast to the profound effects on the serum proteins which are caused by general or specific amino acid deficiencies, the changes in serum protein distribution shown in phenylketonuric individuals with high levels of serum phenylalanine are minor. The excessive serum phenylalanine obviously has little or no effect on the biosynthesis of most of the serum proteins. Several hundred analyses of serum samples from normal and diseased individuals have been made in this laboratory over a period of some years. A review of these disclosed only one which resembled the anomalous pattern of β-lipoproteins seen in most of the younger phenylketonurics. This was from an individual with an *active* case of acute hepatitis. This suggests that there is occasionally a disturbance of liver function in phenylketonuria, in addition to the inability to oxidize phenylalanine to tyrosine in a normal fashion(18), which appears to be the primary inherited biochemical lesion in this disorder. Indications that impaired liver function might occur in phenylketonuria have appeared previously. Coquet, *et al.*(19) observed a perilobular fatty degeneration in the liver of a patient at autopsy and Cowie(20) reported histological abnormalities found in a liver biopsy. Delay, *et al.*(21) found that a galactose tolerance test on a patient with phenylketonuria gave results similar to that found with hepatitis, and Cowie(20) observed an impaired liver function in some, but not all patients, as assessed by an intravenous galactose tolerance test. Furthermore, serums from the patients studied here were frequently strongly colored, indicating a moderately increased icteric index. Evidence that abnormal serum proteins occur as the result of a functional disturbance is afforded by the finding that when abnormalities are present, they disappear after the patients are kept on diets restricted in phenylalanine (F.D., J.Du., R. Du. and M.J.), and, in the one individual who has been followed after restoration of a natural diet, the abnormality has reappeared.

Summary. 1. The greatly increased blood levels of phenylalanine that occur in phenylketonuria do not cause a significant quantitative alteration from normal of the serum protein pattern. 2. Occasional serums of children with phenylketonuria contain abnormal β-globulins. These abnormalities disappear when they are fed phenylalanine-low diets and the blood phenylalanine is lowered to the normal range. There appears to be no correlation between the presence of the abnormal β-globulins and the degree of mental impairment. 3. It is suggested that the abnormal globulins are manifestations of slight liver dysfunction.

We are indebted to Dr. Frank H. Tyler for his assistance in the care of the children maintained in the metabolic ward, and to the administration of Utah State Training School, American Fork, Utah for their aid in obtaining material from other patients. We wish to acknowledge the assistance of Patricia Wall in these studies.

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Glucocorticoid and Mineralocorticoid Activities of Δ^1-Fluorohydrocortisone. (21814)

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During the past 2 years chemical modification of hydrocortisone has resulted in significant changes in biological activity of the hormone. Fried and Sabo(1,2) showed that halogenation at C-9 markedly increases the glycogen deposition activity, while other workers(3-5) found that it causes relatively greater increases in mineralocorticoid potency.

A second hydrocortisone analogue currently under study is the 1-dehydro compound (11β, 17α, 21-trihydroxy-1, 4-pregnadiene-3,20-dione). This compound has antiarthritic activity in man superior to hydrocortisone(6) and greater potency as a glucocorticoid in laboratory animals(7). Enhancement of glucocorticoid activity is not accompanied by a proportional increase in mineralocorticoid potency.

Since 2 such widely differing modifications of the hydrocortisone molecule cause enhancement of glucocorticoid potency, it was of interest to examine the biological characteristics of a compound having both modifications in the same molecule. This report presents the results of glycogen deposition assays and sodium retention tests on Δ^1-fluorohydrocortisone acetate (9α-fluoro-11β,17α, 21-trihydroxy-1, 4-pregnadiene-3,20-dione-21-acetate). The compound is about 50 times as potent as hydrocortisone in the liver glycogen test and almost 5 times as potent as desoxycorticosterone acetate in causing sodium retention in the adrenalectomized rat.

Methods and materials. Steroids. The steroids used were administered as suspensions in an aqueous system of .5% carboxymethyl cellulose, .4% Tween 80, 1.5% benzyl alcohol, and .9% sodium chloride. Suspension was accomplished by grinding crystals and vehicle together in a ground glass tissue homogenizer. The compounds* used were: 11β, 17α, 21-trihydroxy-4-pregnene-3,20-dione (hydrocortisone), 9 α-fluoro-11β, 17α, 21-trihydroxy-4-pregnene-3,20-dione (fluorohydrocortisone), 11β, 17α, 21-trihydroxy-1, 4-pregnadiene-3,20-dione (Δ^1-hydrocortisone), and 9α-fluoro-11β, 17α, 21-trihydroxy-1, 4-pregnadiene-3,20-dione (Δ^1-fluorohydrocortisone). These compounds were used both as the free alcohols and as the 21-acetates. *Animals.* Male rats from the Upjohn colony (Sprague-Dawley ancestry) were adrenalectomized at a body weight of 140-160 g and fed stock laboratory diet (Archer dog pellets) and 1% NaCl as drinking fluid. *Glycogen Deposition Test.* On the morning of the fourth postoperative day food was withdrawn. On the morning of the fifth day the drinking fluid was withdrawn and the rats given the test compound by a single subcutaneous injection of .5 ml of suspended steroid in appropriate dosage. Seven hours later the rats

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* The steroids were generously provided by the Dept. of Chemistry, The Upjohn Co.

TABLE I. Liver Glycogen Deposition Assays of Hydrocortisone, Fluorohydrocortisone Acetate, Δ^1-Hydrocortisone, and Δ^1-Fluorohydrocortisone Acetate.

Assay		Dose (mg)	No. rats	Mean liver glycogen (%)	Potency* (× hydrocortisone)
1	Hydrocortisone	.3	5	.6	
		.6	4	1.2	
		.9	5	2.1	
	Δ^1-Hydrocortisone	.1	5	1.0	
		.2	4	1.1	2.8 (2.6–3.1)
		.3	5	1.7	
2	Hydrocortisone	.3	4	.5	
		.6	5	1.0	
		.9	5	1.8	
	Δ^1-Hydrocortisone	.1	5	.9	
		.2	5	1.2	3.4 (2.8–4.1)
		.3	6	1.4	
3	Hydrocortisone	.3	5	.5	
		.6	5	1.0	
		.9	5	1.7	
	Δ^1-Hydrocortisone	.1	5	.8	
		.2	6	.8	2.5 (2.4–2.7)
		.3	6	1.2	
4	Hydrocortisone	.3	5	.5	
		.6	5	1.2	
		.9	5	1.9	
	Δ^1-Fluorohydrocortisone acetate	.01	5	1.2	
		.03	5	2.0	46.4 (38.6–55.8)
		.09	5	2.5	
5	Hydrocortisone	.3	5	.5	
		.6	5	1.2	
		.9	5	1.9	
	Δ^1-Fluorohydrocortisone acetate	.006	5	.7	48.4 (45.0–52.3)
		.018	5	1.6	
6	Hydrocortisone	.3	5	.6	
		.6	5	1.4	
		.9	4	1.9	
	Δ^1-Fluorohydrocortisone acetate	.005	5	.6	
		.010	5	1.4	53.0 (47.6–59.2)
		.015	4	1.5	
	Fluorohydrocortisone acetate	.025	5	.8	
		.050	5	1.5	12.6 (11.4–13.9)
		.075	4	1.8	

* Figures in parentheses are limits of error at $P = 0.95$.

were anesthetized by intraperitoneal injection of 1 ml 1% Na Cyclopal, the livers removed, weighed, dropped into flasks containing 10 ml hot 30% potassium hydroxide, and digested on a hot plate. The digest was diluted to 100 ml and a 50-fold dilution of an aliquot made for analysis. The anthrone color reaction of Seifter and Dayton(8) was used to estimate glycogen. Ten ml .2% anthrone in 95% sulfuric acid was added to 5 ml of liver digest dilution slowly with cooling; the mixture was heated in a boiling water bath for 10 minutes and optical density measured in a spectrophotometer at 620 $m\mu$. Calculation of glycogen content was made by comparison with standards of glucose. Experimental design was to give 3 doses of any compound to groups of 4 to 6 rats; at least 2 compounds were always tested simultaneously in this manner. Potency ratios and limits of error were calculated by the method of Irwin(9). Hydrocortisone was used as the reference standard; all potencies are expressed in terms of this compound. *Sodium Retention Test.* This test, a modification of the method of Marcus, Romanoff, and Pincus(10), was car-

TABLE II. Sodium Retention Activity of Hydrocortisone Acetate, Fluorohydrocortisone Acetate, Δ^1-Hydrocortisone, Δ^1-Hydrocortisone Acetate, and Δ^1-Fluorohydrocortisone Acetate.

Compound	Dose (μg)	No. rats	Sodium excretion (% of control)	Potency (× DOCA)
DOCA	1	24	69 (58– 84)	
	2.5	6	59	
	5	12	48 (40– 55)	1.0
	10	126	48 (32– 70)	
	20	12	35 (32– 38)	
	40	12	27 (26– 28)	
Hydrocortisone acetate	5,000	6	83	
	10,000	6	80	—*
	20,000	6	73	
Fluorohydrocortisone acetate	0.1	12	90 (84– 95)	
	0.2	6	80	
	0.4	6	70	
	0.625	6	42	
	1	60	53 (42– 70)	
	1.25	12	50 (42– 57)	5.0
	1.5	6	56	
	2	6	48	
	2.5	12	40 (38– 42)	
	3	6	31	
	4	6	41	
	5	30	37 (33– 41)	
Δ^1-Hydrocortisone	1	6	92	
	100	6	85	—†
	10,000	6	70	
Δ^1-Hydrocortisone acetate	1	6	105	
	10	6	107	—
	100	6	110	
	1,000	6	108	
	10,000	12	100 (85–115)	
Δ^1-Fluorohydrocortisone acetate	0.25	6	74	
	0.5	6	64	4.6
	1	12	52 (50– 53)	

* No evidence of sodium retention at doses below 5 mg. Activity at high doses is so slight that no calculation of potency can be made.

† Although no calculation of potency was made, very slight tendency toward sodium retention is observed as dosage increases.

ried out as follows: On the morning of the fourth postoperative day food and drinking fluid were withdrawn. On the following day each rat was given 5 ml water by stomach tube; one hour later it was given 5 ml .9% NaCl orally and injected with the test compound suspended in .2 ml of the vehicle. Control rats were similarly treated but received injections of the vehicle alone. The rats were lightly etherized to induce emptying of the bladder and placed in metabolism cages, 2 rats per cage, 3 cages per dosage group, for 4 hours, and again etherized and removed from the cages. Urine volume was recorded and cages rinsed over the collection cylinders with a distilled water spray. Collections were diluted to 100 ml and appropriate dilutions analyzed for sodium with a flame photometer. Sodium excretion is expressed as percent of excretion of control animals. The *experimental design* was similar from one test to another, *i.e.*, multi-dose comparisons of compounds. The reference standard was desoxycorticosterone acetate (DOCA), but in some experiments is was more expedient to compare one hydrocortisone analogue with another analogue than with DOCA. Since interpretation is simpler if one refers the potency of the hydrocortisone analogues to DOCA, the data were pooled and submitted to an Irwin (9) analysis of potency ratios with respect to DOCA.

Results. Table I presents the results of the glycogen deposition assays. The mean value

(3 assays) for potency of Δ^1-fluorohydrocortisone acetate is 49.3 times hydrocortisone. Δ^1-Hydrocortisone is (3 assays) 2.9 times hydrocortisone, while fluorohydrocortisone (1 assay) is 12.6 times hydrocortisone.

Table II presents sodium retention data on Δ^1-hydrocortisone and on the acetates of hydrocortisone, fluorohydrocortisone, Δ^1-hydrocortisone, and Δ^1-fluorohydrocortisone. Hydrocortisone acetate is virtually inert in this biological system, and reference of activity to this compound is impossible—hence the use of the "secondary standard," DOCA. Fluorohydrocortisone acetate is 5 times as potent as DOCA, Δ^1-hydrocortisone acetate is almost inactive, and Δ^1-fluorohydrocortisone acetate is 4.6 times as potent as DOCA.

Discussion. The glucocorticoid activity of fluorohydrocortisone has been reported by others(1) to be several times that of hydrocortisone. Its anti-inflammatory potency has been reported to be 13 times that of hydrocortisone(11). The reported activity varies somewhat from one laboratory to another, but the currently reported figure of a 12-fold enhancement is compatible with the other data.

Bunim and Pechet(6) have indicated that Δ^1-hydrocortisone is 2 to 4 times as active as cortisone in causing alleviation of rheumatoid arthritis. Perlman and Tolksdorf(7) found a ratio of 3.9 for Δ^1-hydrocortisone/hydrocortisone in the liver glycogen deposition test. These factors are not widely divergent from our finding that Δ^1-hydrocortisone is 3 times as active as hydrocortisone in glycogen activity.

The electrolyte activities of the compounds studied deserve some comment. In our test system hydrocortisone is almost inactive, tremendous doses being required to cause significant retention of sodium. Δ^1-Hydrocortisone is also nearly inert as far as sodium retention is concerned. Substitution of the hydrogen atom at C-9 with a fluorine atom in these compounds produces potent sodium retaining agents.

It is significant that Δ^1-fluorohydrocortisone is 4 times as potent as fluorohydrocortisone in the glycogen deposition test, but is no more potent than fluorohydrocortisone in the sodium retention test.

Summary. (1) Comparisons have been made of relative potencies of hydrocortisone, fluorohydrocortisone, Δ^1-hydrocortisone, and Δ^1-fluorohydrocortisone in causing deposition of liver glycogen and reduction of sodium excretion in the adrenalectomized rat. (2) In glycogen deposition potency, the compounds have activity of the following magnitude: Δ^1-fluorohydrocortisone acetate—50; fluorohydrocortisone acetate—12.6; Δ^1-hydrocortisone—2.9; hydrocortisone—1. (3) In sodium-retaining potency, the compounds have activity of the following magnitude: Δ^1-fluorohydrocortisone acetate—4.6; fluorohydrocortisone acetate—5.0; Δ^1-hydrocortisone acetate—very slight; hydrocortisone acetate—very slight; DOCA—1.

Addendum. Since the original submission of this report to this journal Hirschmann, Miller, Beyler, Sarett, and Tishler have published the preparation of Δ^1-fluorohydrocortisone acetate (*J. Am. Chem. Soc.*, 1955, v77, 3166). They find that in a mouse liver glycogen deposition test and in a rat granuloma-inhibition test the compound is 25 times as potent as hydrocortisone acetate.

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Vitamin B_{12}: Correlation of Serum Concentrations and Age. (21815)

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Vit. B_{12} occupies an important place in the nutrition of microorganisms, lower animals, and man, but as yet neither minimal nor optimal daily requirements of this vitamin have been established for humans. Accordingly, studies of the distribution of vit. B_{12} in tissues and circulation of healthy and diseased persons seem justified. Previous studies have employed assay methods that raise the question as to whether total vit. B_{12} was measured, or they have dealt with a limited number of patients. The present investigation was therefore directed toward (a) evaluation of the *Lactobacillus leichmannii* assay method when applied to serum and (b) accumulation of sufficient data in "normal" individuals of various ages to justify statistical treatment of the results obtained. All persons reported in this study were free of overt laboratory or physical evidence of disease, and all were ambulatory. Individuals with cardiac, hepatic, renal, or pulmonary disease were eliminated. Estimations of serum vit. B_{12} were performed on more than 1000 persons, but, after eliminating all subjects with some deviation from "normal" health, 528 individuals constitute the series reported here. Microbiological methods for the determination of vit. B_{12} which require a turbidimetric measurement of the response to vit. B_{12}, such as is essential in the *Euglena gracilis* method(1,2), require clear and colorless samples for assay. Methods for the determination of vit. B_{12} where a titrimetric response is employed, such as the *L. leichmannii* method(3), permit the direct assay of turbid and colored solutions. Preliminary to the present studies, a number of serum samples were assayed by the *L. leichmannii* assay (a) after clarification by centrifugation following heat denaturation of the serum proteins near their isoelectric point and (b) after simple dilution without preliminary removal of proteins. In the assay of "clarified" samples the denatured proteins are *discarded* and do not come in contact with the assay organism, whereas in the assay of "diluted" samples coagulation of the serum proteins takes place directly in the culture tubes. These preliminary studies demonstrated that the results obtained on the "clarified" samples were considerably lower than those obtained with the "diluted" samples. Vit. B_{12} added to serum prior to clarification could not be recovered quantitatively, as noted by previous investigators, whereas vit. B_{12} added to serum prior to dilution could be quantitatively accounted for. Apparently even heat denatured proteins are capable of combining with, or adsorbing, the vitamin, since the values obtained from *clarified* samples of heat coagulated sera were low with respect to original vit. B_{12} as well as to added vit. B_{12}. The results suggest the desirability of distinguishing clearly between (a) the usually small amount of "free" vit. B_{12} present in serum, (b) the major portion of vit. B_{12} bound dynamically to blood proteins, chiefly to alpha globulin(4), that can be rendered free for microbiological assay by protein denaturation and (c) vit. B_{12} adsorbed onto heat coagulated protein. This physically adsorbed vit. B_{12} seems to have been frequently overlooked. Discrepancies in results seem to be a matter of differences in sample preparation, rather than one of inability of either organism to measure the vitamin. When the same method of preparing samples is employed, the results obtained with the two organisms are in good agreement(5-7). Accordingly, criticism of *L. leichmannii* as a test organism(8) seems unjustified.

Materials and methods. The patients studied are classified into Groups A, B, C, and D. Group A, 108 males, and Group B, 238 females, were derived from the Norristown State Hospital. All patients were ambulatory, had been inmates for 6 months or more; all had well-balanced *ad libitum* diets, and none of the patients studied had eating patterns that were markedly abnormal. Vitamin supplements were not available for chance ingestion and none were administered during the period of study. Samples from both males and females were obtained in sufficient number to give statistical validity to any difference observed. There was no selection of patients other than to insure sampling at all age levels of the population represented in the hospital. As Groups A and B represented an institutionalized population that might have unique and unsuspected characteristics, other populations were sampled. Group C was derived from the Vineland State School in Vineland, N. J. The authors are indebted to Dr. Miles Drake for his cooperation in obtaining samples from 41 females at this institution. The same criteria of "normal health" were observed, and in this institution also an *ad libitum* well-balanced diet was available. Group D represents ambulatory male blood donors from Baltimore, Md. In keeping with the regulations of all blood-donation centers, these individuals were examined and found to be free of overt disease. Information as to prior dietary habits and vitamin supplementation are unknown, but these circumstances may be presumed to obtain for the population at large. This group of 141 males might therefore be regarded as a contrast to the institutionalized Groups A, B, and C. The authors are indebted to Dr. Robert E. Bauer, of the University of Maryland School of Medicine, for his cooperation in obtaining these sera. Blood samples were drawn from all patients in the fasting state (9). Bloods were allowed to clot, and the sera were harvested into 10-cc sterile serum vials which were refrigerated at −4°C until assayed for total vit. B_{12} content. The specimens from Vineland and Baltimore were packed in dry ice and carried to the laboratory. All specimens were assayed under code, without knowledge of age, sex, or health of the patient from whom the specimens had been obtained. Vit. B_{12} was determined by microbiological assay with *L. leichmannii*, according to the method of Skeggs *et al.*(3). Guanylic acid was used routinely in the basal medium at 0.25 mg per ml of single strength medium. Recrystallized thiomalic acid, at 0.50 mg per ml of single-strength medium, was the reducing agent employed. Crystalline vit. B_{12} at levels of 0, 0.02, 0.04, 0.06, 0.08, 0.12 and 0.20 $m\mu g$ was used as the standard. Except as noted, suitable aliquots (usually 1, 2, 3, and 5 ml) of a 1:20 dilution of serum were assayed. Standard as well as appropriate dilutions of serum (or protein-free filtrates of serum, where specifically indicated) were autoclaved *directly with the assay me-*

TABLE I. Recovery Experiments with Vit. B_{12}.

Method of preparing serum for assay			
Heat clarified* ($m\gamma$/ml)		Not heat clarified† ($m\gamma$/ml)	
	%		%
.43		.72	100
.35		.67	102
.20		.45	107
.17		.53	103
.22	83	.86	
.12	57	.50	
.25	63	.74	
.26	57	.68	
.31		.80	
.31		.72	
.41		.74	
.37		.98	
.60		1.75	
.11		.51	
.25		.88	
.19		.48	

* Heat-clarified serum prepared for assay as follows: 2 ml serum, 2 ml 0.15 M acetate buffer of pH 4.7, 6 ml water, all in a test tube capped and heated in a boiling water bath for 30 min., centrifuged, and the supernatant decanted, pH of supernatant adjusted to 6-7 with 0.1 N NaOH, suitable aliquots pipetted into tubes for assay. Where recoveries were done, 6 ml of an 0.0001 γ of vit. B_{12}/ml solution replaced the 6 ml of water.

† Unheated serum prepared for assay as follows: 1 ml of serum diluted to 50 ml with water, suitable aliquots pipetted into tubes for assay. Where recoveries were done, 2 ml of an 0.001 γ of vit. B_{12}/ml solution replaced 2 ml of water.

Percent figures indicate the extent to which vit. B_{12} added to serum as indicated above could be recovered.

TABLE II. Summary of Sample Statistics.

Group	No. of subjects	Avg age	Avg B_{12} blood level, $m\gamma$/ml	Regression coefficient	Significance of regression
A	108	42	.58	0	—
B	238	59	.57	−.0022	$P < .05$
C	41	37	.70	−.0052	$P < .001$
D	141	32	.51	−.0048	$P < .01$

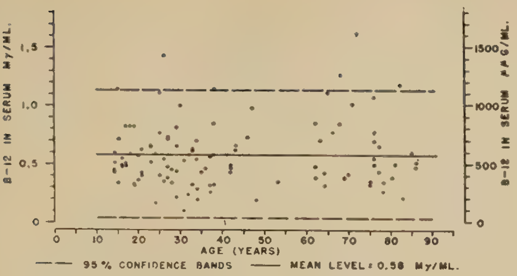

FIG. 1. Serum concentration of vit. B_{12} as a function of age in Group A (108 males).

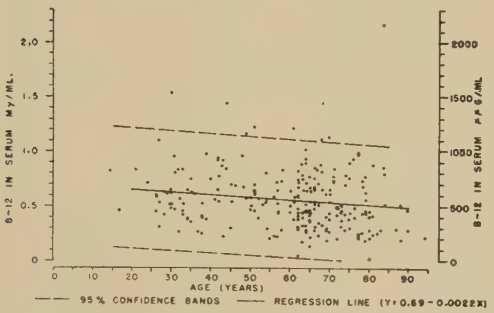

FIG. 2. Serum concentration of vit. B_{12} as a function of age in Group B (238 females).

dium. Titration of acid production after an incubation period of 72 hours was used to determine the extent of bacterial growth in response to vit. B_{12}.

Results. The results of a study of 2 methods for the preparation of serum samples for assay are summarized in Table I. The results obtained on "diluted" samples are significantly higher than those obtained on "clarified" samples. Recovery of vit. B_{12} was found to be quantitative in the former instance and generally low and variable in the latter. These results are offered in justification for the method selected for this study.

The results are presented in Table II and Fig. 1 to 4. The total serum vit. B_{12} content is plotted against age. The data derived from the respective groups were submitted to statistical analysis. In Group A there was no regression of serum vit. B_{12} concentration with age, the mean value being 0.58 $m\mu g$ per ml. In Groups B, C and D there were significant regressions with age, the regression lines for the respective groups are $Y = 0.69—0.0022X$, $Y = 0.89—0.0052X$, and $Y = 0.67—0.0048X$. It is noteworthy that all of the values falling outside of the 95% confidence limits in 3 of the 4 groups are *high values.* When high values were observed, the hospital records were rechecked to determine that the patients had received neither special vitamin therapies nor peculiar diets high in vit. B_{12} content. Further, additional blood samples were drawn and assayed for vit. B_{12} content. The duplication of values was striking and gave indication of some constancy to the circulating vit. B_{12} in the same individual. Although every attempt was made to discover a cause for these high values, no explanation was forthcoming. Other investigators have also noted high values(2, 6,10,11,12,13), and subsequent work is indicated to explain these findings. Fig. 5 presents a frequency histogram of the serum vit. B_{12} concentrations observed in Group A. The skewness of the observed distribution confirms the fact that these few high values are distinctly outside the range of normal expectancy, provided there is justification for assuming that serum B_{12} concentrations should be normally distributed.

Discussion. From the foregoing data it is apparent that the total circulating vit. B_{12} in man varies considerably from patient to patient at all age levels and that there is a gen-

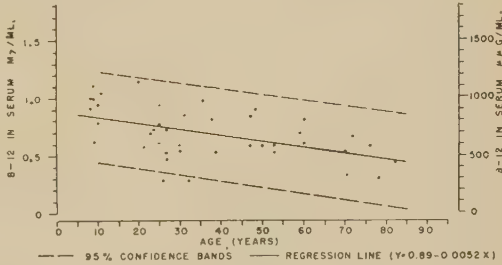

FIG. 3. Serum concentration of vit. B_{12} as a function of age in Group C (41 females).

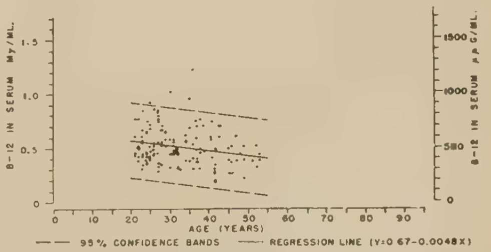

FIG. 4. Serum concentration of vit. B_{12} as a function of age in Group D (141 males).

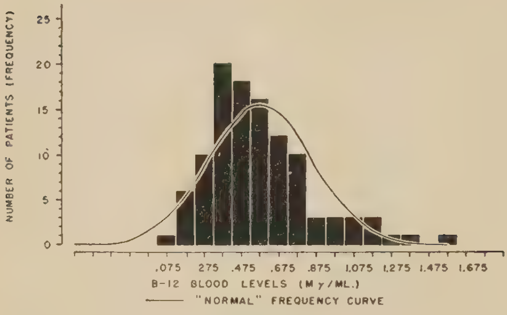

FIG. 5. Frequency histogram of serum B_{12} concentration in 108 males (Group A).

eral trend toward lower levels in advanced age. The wide range of individual values has been previously noted(6,14,15), and the decline in values with age has been commented upon(16-18). The values observed in this study tend to be higher than elsewhere reported but they are in good agreement with results obtained with *L. leichmannii*(6) and *E. gracilis*(7,19) methods when *diluted* specimens were assayed.

While the present studies have shown that in a large group of "normal" individuals there is a trend toward reduction in total circulating vit. B_{12} with age, the real biological significance of the results is in doubt. Subsequent investigations will be directed toward discovering whether particular abnormal groups, *i.e.*, hypertensives, diabetics, and cirrhotics, have total vit. B_{12} concentrations differing significantly from those of this "normal" group. Such differences have been reported(12,20).

Summary. Total serum vit. B_{12} concentrations were determined in 528 individuals by the *L. leichmannii* method, and a justification for the choice of this method of assay is presented. The individuals studied were "normal" as far as physical health is concerned, and the major variable was age. In such persons there is a trend toward lower serum concentration of vit. B_{12} in the aged than in younger age groups. Although this difference is statistically significant, the biological importance of this finding is not apparent. There is wide individual difference of values in serum vit. B_{12} concentration at all age levels, but in this "normal" group values below 200 μμg/ml and above 1000 μμg/ml were uncommon. The average for the entire 528 individuals was 560 μμg/ml, the 95% confidence bands being 70 and 1060 μμg/ml respectively.

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Cytopathogenic Effect of Newcastle Virus in Monkey Kidney Cultures and Interference with Poliomyelitis Viruses.* (21816)

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During the course of experiments on variants of poliomyelitis virus which might be capable of multiplication in chick embryo tissue culture, another agent which produced a cytopathogenic effect on epithelial cells in monkey kidney cultures was encountered. This agent was identified as Newcastle disease virus (NDV) and was traced to the chicken plasma used in the chick embryo explant cultures. The purpose of this communication is to describe the behavior of this virus in monkey kidney cultures and its interfering effect on the cytopathogenic change produced by poliomyelitis and certain human "enteric" viruses.

Materials and methods. The chick embryo explant cultures in roller tubes were prepared with plasma derived from chickens purchased in the market. The culture fluid consisted of 40% horse serum heated at 56°C for 30 minutes, 40% Earle's solution, and 20% chick embryo extract diluted 1:20. After about 4 days when a good growth of fibroblasts had been obtained, this medium was replaced by another consisting of 0.5% lactalbumin hydrolysate in Earle's solution with or without 5% heated horse serum. The monkey kidney cultures consisted of an outgrowth of trypsinized cells in roller tubes maintained in a medium of 0.5% lactalbumin hydrolysate in Earle's solution and 0.5% heated calf serum. The hemagglutination tests were performed with 0.5% chick erythrocytes using 0.02 M phosphate buffer pH 7.0 in physiologic salt solution. The patterns were read after sedimentation at 4°C for 40 minutes. Chicken antiserum for the B strain of NDV and rabbit antiserum for the Cg. strain of NDV were kindly supplied by Dr. Frederik B. Bang. The poliomyelitis viruses used in these tests were special derivatives of the Mahoney, YSK, and Leon strains(1) and the human "enteric" viruses were derived from healthy children in Cincinnati(2).

Results. Isolation and identification of agent from chick embryo tissue cultures. Agents producing a cytopathogenic effect in monkey kidney cultures quite distinct from that produced by the poliomyelitis viruses were recovered from the fluids of 4 separate series of chick embryo cultures seeded with poliomyelitis virus as well as from control cultures to which no virus had been added. It is noteworthy that the fibroblasts in the chick embryo cultures which yielded this agent underwent degeneration in 6 to 8 days while in 2 series of cultures from which no such agent was recovered no degeneration of the fibroblasts occurred. Investigation of the various components of the chick embryo tissue culture system revealed that the chicken plasma which had been stored for 7 months at 4°C contained the agent. Two isolations from chicken plasma in monkey kidney culture and one in 10-day embryonated eggs established this fact. Allantoic inoculation of 10-day eggs was followed by death in 2 to 3 days with hemorrhagic lesions in the embryos. Allantoic fluid from these eggs yielded a hemagglutinin for chick erythrocytes in a titer of 1:128 which eluted rapidly at room temperature. The agent was identified as NDV by hemagglutination-inhibition (Table I), and also by a test in monkey kidney tissue cultures in which 200 TCD_{50} of the virus were neutralized by the chicken antiserum in a dilution of 1:400 and by the rabbit antiserum in a dilution of 1:6, while undiluted normal chicken and rabbit sera were without effect; all sera were heated at 56°C for 30 minutes.

Properties of NDV in monkey kidney tissue culture. Monkey kidney epithelial cells affected by NDV virus first exhibit a dense

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* Aided by a grant from the National Foundation for Infantile Paralysis.

TABLE I. Identification of Agent Recovered from Chick Embryo Tissue Cultures and from Chicken Plasma.

Agent		Hemagglutination-inhibition serum titer vs. 8 units antigen	
Source	Passage	Normal chicken	NDV immune chicken
Chick embryo tissue culture	Monkey k* 1 Egg 1	<5	640†
Chicken plasma	Egg 1	"	1280
	Monkey k 2 Egg 1	"	1280

* k = kidney.

† Reciprocal of initial serum dilution.

granularity in the cytoplasm which is often followed by a swelling of the cell. Affected cells break away from the epithelial sheet and gradually disintegrate. In tissue cultures fixed in Bouin's solution and stained by hematoxylin and eosin one can also distinguish occasional syncytial masses containing 2 to 5 nuclei similar to those described in HeLa cells affected by NDV and mumps virus(3). The time required for these cytopathogenic effects to appear was 1 to 6 days depending upon the concentration of NDV.

The lesions, which began as discrete foci in large epithelial sheets, enlarged during the next 24 to 72 hours, but invariably left 10 to 50% of the cells unaffected. The disintegrated cells fell off the glass leaving sheets of various sizes of normal-appearing epithelial cells, which continued to metabolize as evidenced by an acid change in the medium. In some of the tests more epithelial cells were spared in the tubes inoculated with the larger amounts of virus.

The cytopathogenic effect was carried through 6 serial passages in monkey kidney cultures with a total dilution of the original chick culture material of 10^{-22}. When harvested at the height of the degenerative change monkey kidney tissue culture fluid from the 1st and 3rd passages contained a moderately high titer of virus infective for tissue culture ($10^{6.2}$ to $10^{6.5}$ TCD_{50} per ml) and a low titer of hemagglutinin for chick erythrocytes (1:2 to 1:16). Allantoic fluid from NDV infected eggs contained larger amounts of both infective virus and hemagglutinin

TABLE II. Effect of Poliomyelitis and Certain Human "Enteric" Viruses on Monkey Kidney Epithelial Cells Which Escaped Destruction by NDV.

Challenge virus 10^3 TCD_{50}	Treatment of cells 4 days prior to challenge	% of cells exhibiting characteristic cytopathogenic change in different tubes on indicated day after addition of challenge virus					
		1	2	3	4	5	6
Poliomyelitis Type 1 (Mahoney, KP35)	NDV	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 10	0, 0, 0, 0, 0	0, 0, 0, 5, 10
	0	5, 5, 10, 15	100, 100, 100, 100				
Poliomyelitis Type 2 (YSK, KP53)	NDV	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 10	0, 0, 0, 1, 25
	0	0, 1, 5, 5	75, 75, 75, 100	100, 100, 100, 100			
Poliomyelitis Type 3 (Leon, KP36)	NDV	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 1	0, 1, 5, 5, 10	0, 5, 25, 25, 40
	0	0, 1, 1, 5	50, 60, 60, 75	100, 100, 100, 100			
Human enteric Type 1 (Wallace, KP3)	NDV	0, 0, 0, 0, 0	0, 0, 0, 0, 0	20, 25, 30, 30, 30	60, 75, 85, 95, 100	95, 100, 100, 100, 100	100, 100, 100, 100, 100
	0	10, 15, 30, 50	100, 100, 100, 100				
Human enteric Type 2 (Bryson, KP4)	NDV	0, 0, 0, 0, 0	0, 0, 0, 0, 0	5, 25, 30, 30, 30	50, 50, 75, 75, 90	90, 95, 95, 95, 95	100, 100, 100, 100, 100
	0	25, 30, 50, 50	85, 85, 100, 100	100, 100, 100, 100			
Human enteric Type 3 (Hill, KP7)	NDV	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 1
	0	5, 10, 10	60, 60, 85	85, 100, 100	100, 100, 100		
Human enteric Type 5 (Gregory, KP5)	NDV	0, 0, 0, 0, 0	0, 0, 0, 0, 0	5, 10, 10, 25, 25	10, 25, 60, 80, 100	0, 0, 80, 95, 100	10, 10, 90, 100, 100
	0	10, 25, 30	75, 75, 85	100, 100, 100			

5 roller tubes containing about 30 to 50% of residual cells following infection with about $10^{4.5}$ TCD_{50} of NDV were used for each challenge virus. The original epithelial sheet in these tubes covered an area of about 4 × 1.5 cm. Untreated tubes of the same series were used for control.

($10^{8.2}$ TCD_{50} per ml and 1:128 to 1:256 respectively). *Interference with poliomyelitis and human "enteric" viruses in tissue culture.* It was important to determine whether the accidental presence of NDV in a chick embryo or mammalian tissue culture system might interfere with poliomyelitis or other viruses. Evidence for interference was sought by challenging the monkey kidney epithelial cells which escaped destruction by NDV infection with each of the 3 types of poliomyelitis virus and with 4 of the human "enteric" prototype viruses. The results shown in Table II indicate that the normal-appearing residual cells were resistant to the cytopathogenic effect of all 3 types of poliomyelitis virus and of the Type 3 human "enteric" virus. A partial resistance characterized by delay in appearance of cytopathogenic effect was observed with the Type 1, 2, and 5 human "enteric" viruses. In some instances notably with Type 1 poliomyelitis and Type 5 human "enteric" virus the degenerative change seen in a small proportion of the NDV residual cells was not progressive. The affected cells fell off the glass and the culture resumed its normal appearance.

Discussion. Growth of NDV with cytopathogenic change in chick embryo fibroblasts (4,5), chick embryo epithelial cells of amniotic origin(5), and HeLa cells(3,6) has been reported but no mention was made of serial passage in these cell systems. In the present studies NDV was serially propagated in monkey kidney epithelial cultures, producing a characteristic cytopathogenic effect and multiplying to a high level.

The present demonstration that NDV can be propagated in series in monkey kidney cultures with the production of a cytopathogenic effect in a varying proportion of the cells points to the need of eliminating the presence of this virus in any tissue culture system employing chicken plasma for explanted tissues. The fact that monkey kidney epithelial cells which escaped destruction by NDV exhibited varying degrees of resistance to the cytopathogenic effect produced by the antigenically unrelated poliomyelitis and human enteric viruses, adds still another virus to those previously reported as inducing varying degrees of resistance in cells visibly unaffected by the "initial" viral experience(7,8,9). Future studies will have to establish whether the interference involves only cellular disintegration or viral multiplication as well.

Summary. Newcastle disease virus accidentally encountered in chicken plasma used as a tissue culture reagent was serially propagated in monkey kidney epithelial cells with the production of granular degeneration and disintegration. Ten to 50% of these cells escaped the cytopathogenic effect of the virus and were then found to be almost completely resistant to the cytopathogenic effect of the 3 types of poliomyelitis and Type 3 human "enteric" viruses; partial resistance as evidenced by a delay in appearance of cytopathogenic change was demonstrated for Types 1, 2, and 5 human "enteric" viruses.

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Filament Induction in *Escherichia coli* by Ricinoleic Acid.* (21817)

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A variety of compounds have been reported (1-5) to interfere with cell division without affecting the synthesis of cell substance. The cultivation of bacteria in the presence of such compounds results in the development of the organisms as elongated filaments. As part of an investigation of the effect of lipids on cell division, we have observed consistent filament formation in *Escherichia coli* when cultivated in media containing ricinoleic acid (12-hydroxy-9:10-octadecenoic acid). A number of structural variants of this fatty acid, as well as related compounds, have been studied to determine their effect on the growth pattern of this organism.

Materials and methods. A laboratory strain of *E. coli* was employed. After growth overnight on nutrient agar, the organisms were removed and suspended in saline to a density of 200 on a Klett-Summerson colorimeter (KS filter no. 42). One ml portions of this suspension were employed as inocula for flasks containing 100 ml of 0.5% of proteose-peptone medium. The flasks were suspended in a 37°C water bath, and were equipped for the periodic addition of sterile 1N NaOH so that the pH of the medium could be maintained in the range 7.6 to 7.8. A Beckman model K automatic titrator was adapted for this purpose. During the growth cycle the medium was agitated by means of a magnetic stirring bar. For routine checking of the effect of the various fatty acids on the morphology of the organisms, samples were removed at hourly intervals and stained with safranine. For special studies, nuclear staining was performed as described by Robinow (6), and cell walls were stained by the method of Webb(7). The various fatty acids were studied over a concentration range of 1 to 5 mg/ml medium. Commercial ricinoleic acid was purified repeatedly by a variation of the method of Kozlowski(8). Ricinelaidic acid was prepared as described by Griffiths and Hilditch(9), and 12-keto-oleic acid by the method of Ellis(10). The other acids were commercial products and used as purchased.

Results. Effect of pH on filament formation. In confirmation of the results reported by Henrici(5), we found that cultivation of *E. coli* on nutrient agar saturated with sodium ricinoleate resulted in the development of filamentous forms during the first hours of logarithmic growth. The filaments soon began to break up, however, and by the end of the log phase organisms of normal morphology were obtained. When the ricinoleate agar was adjusted initially to pH values ranging from 5-8, it was observed that filament formation was more marked and persisted for longer periods at the more alkaline pH value. Subsequent utilization of liquid proteose-peptone media, with arrangements for periodic readjustment of the pH to the range 7.6-7.8, was found to result in maintenance of the cells in filamentous form throughout the entire growth cycle. Lowering the temperature of incubation below 37°C was found to reduce the tendency toward filament formation. When the inorganic salts medium of Monod and Wollman (11) was employed, sodium ricinoleate was without effect on the morphologic appearance of the organisms. A similar observation has been reported by Webb(12) in which filament formation in Gram positive bacilli was observed to occur when the organisms were grown in magnesium-deficient peptone media, but not in a magnesium-deficient inorganic salts medium.

Fig. 1-3 illustrate the morphology of *E. coli* after incubation in a proteose-peptone medium for 1, 3 and 5 hours respectively. By comparison, Fig. 4-9 demonstrate the continued elongation of the organisms when grown in a medium containing added ricinoleate. Using the cell wall stain of Webb(7) it was found that the filaments were non-sep-

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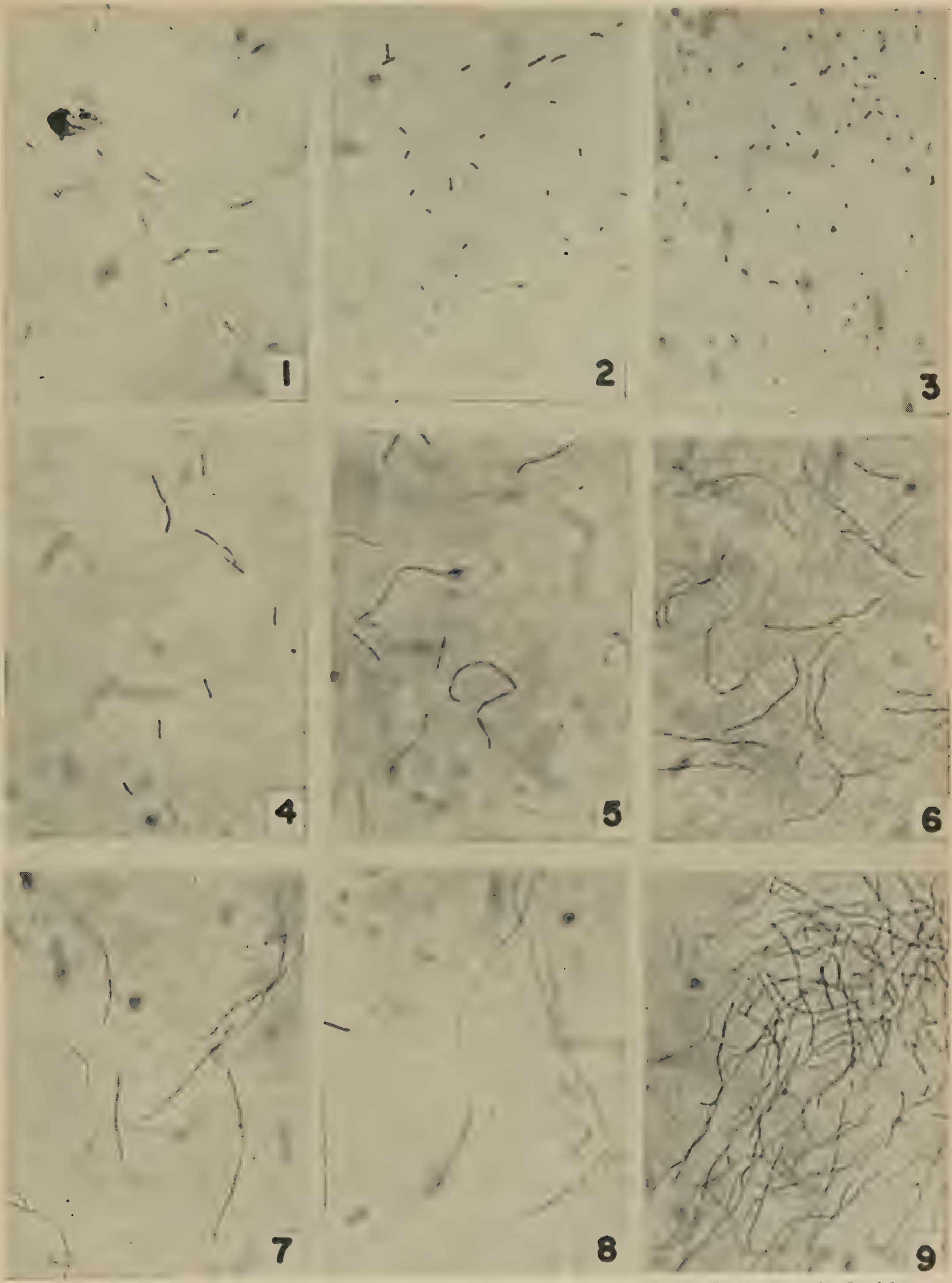

FIG. 1-9. *Escherichia coli* normal forms and filaments produced by ricinoleate, stained with safranine, ×900.

FIG. 1-3. *E. coli*, proteose-peptone medium, 1, 3, 5 hr incubation respectively.

FIG. 4-9. *E. coli*, proteose-peptone medium with sodium ricinoleate 5 mg/ml at 2, 3, 4, 5, 6 and 8 hr incubation respectively.

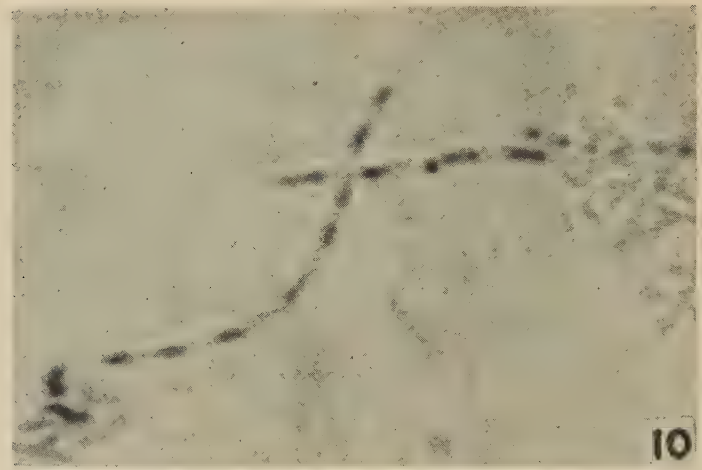

FIG. 10. *E. coli,* 4 hr incubation in ricinoleate-proteose-peptone medium. Nuclear stain. ×3600.

tate. Organisms obtained after 4 hours cultivation in a ricinoleate medium (Fig. 6) were stained by the method of Robinow(6) and shown to be multinucleate (Fig. 10).

Effect of variation in structure of the fatty acid employed. Over the concentration range of 1-5 mg/ml, and under conditions of periodic readjustment of the pH, a number of fatty acids were tested for an ability to inhibit cell division in *E. coli.* The morphology of the organisms was followed throughout the growth cycle. At a level of 5 mg/ml, it was found that added oleate, stearate, 12-hydroxystearte, 12-ketooleate, and ricinelaidiate were without effect on the morphologic appearance of the organisms during any stage of the growth cycle. The failure of these fatty acids to induce filament formation is of special interest since it establishes the importance of both the 9:10 unsaturation and the 12-hydroxy group in ricinoleic acid, as well as the cis configuration around the double bond, as the structural features essential for the effect of this compound on cell division in *E. coli.* As might be expected, lower molecular weight fatty acids such as capric, pelargonic, myristic, and palmitic failed to induce filament formation. *E. coli* also exhibited essentially normal morphology when grown in the presence of a typical synthetic anionic detergent, sodium lauryl sulfate.

Washing the inoculum with saline was found to have a marked effect on filament formation. If the growth from the nutrient agar was suspended in saline for a period of one hour, the cells collected by centrifugation and resuspended in fresh saline for an additional hour before the final inoculum was prepared, filament formation was observed not only in ricinoleate media but also in media containing other fatty acids such as oleic acid. Presumably the saline treatment washed out substances essential to cell division in the organisms, the damaged cells then becoming susceptible to the effect of oleic acid. When the saline extraction was shortened to one 10 minute treatment, only occasional filaments were observed in oleate media. Cowie and Roberts (13) have reported that glucose-1-phosphate, fructose-1, 6-diphosphate, glutamate, cystine, methionine, and glutathione are metabolites which readily diffuse out of *E. coli.* These compounds were added to proteose-peptone-oleate medium at the following concentrations per ml: glucose-1-phosphate 0.5 mg, fructose-1, 6-diphosphate 0.5 mg, glutamate 0.5 mg, cystine 0.4 mg, methionine 0.4 mg, glutathione 0.1 mg. In such a medium oleic acid failed to induce filament formation even if washed inocula were employed. By comparison, ricinoleic acid continued to elicit marked elongation of the organisms.

Effect of various compounds on division of the filaments. Hotchkiss(14) has presented evidence for the production of cytolytic damage in bacteria under the influence of surface active agents. The loss of cell solutes caused by such agents was measured in terms of the percentage of nitrogen and phosphorus compounds released from the organisms, compared with the amounts initially extractable by trichloroacetic acid. Surface active agents which caused the release of a major percentage of cell nitrogen and phosphorus compounds were found to have bactericidal activity. A possible parallel explanation for the effect of ricinoleate on cell division in *E. coli* would be that by specifically altering membrane permeability it permits the loss of cell solutes essential to the division process. The data given above indicate that several metabolic intermediates to which the membrane of *E. coli* is said to be readily permeable, are not involved in the mechanism of action of ricinoleate. To further test this hypothesis *E. coli* was cultivated in a ricinoleate medium for 4-6 hours, the filamentous cells collected by centrifugation and resuspended in solutions of various inorganic or organic compounds. The pH of the suspensions was maintained in the range 7.6-7.8, since a decrease in pH was in itself followed by eventual division of the filaments into organisms of essentially normal morphology. Samples of the suspensions were removed after one hour and 3 hours of incubation in solutions of the compounds being tested. Microscopic observations were then made to determine whether the filaments were retaining their structure, or whether the process of cell division had begun to occur.

The compounds tested, and the concentrations in mg/ml employed were: adenine 0.05; adenosine 0.05; adenylic acid 0.05; adenosine diphosphate 0.05; adenosine triphosphate 0.05; sodium chloride 0.87; potassium chloride 1.1; sodium phosphate 0.01, 0.05, 0.1, 1.0, 4.0; sodium arsenate 0.1, 1.4; glucose-1-phosphate 0.5; fructose-1, 6-diphosphate 0.5; cystine 0.4; methionine 0.4; glutamate 0.5; and glutathione 0.1. Of the compounds tested, only inorganic phosphate consistently caused the filaments to divide, the effect being definitely discernible at 1 hour and complete at the end of the 3 hour period. A concentration of 0.1 mg/ml was required for complete division of the filaments to occur, although a partial effect could be seen at concentrations of 0.05 and 0.01 mg/ml. The fact that sodium arsenate did not initiate cell division, and that the filaments retain their structure in solutions containing both sodium phosphate and ricinoleate, suggests that the mode of action of the fatty acid is in some way concerned with a disturbance in phosphate metabolism.

Summary. Sodium ricinoleate interferes with cell division in *E. coli* causing the organisms to develop as filaments. The effect appears to be specific and dependent upon the 9:10 unsaturation and the 12-hydroxy group as well as the cis-configuration around the double bond, present in this fatty acid. A variety of metabolites, as well as other organic and inorganic compounds, were tested for an ability to induce cell division in filamentous forms of the organisms. Of the substances evaluated, only inorganic phosphate consistently and rapidly caused such an effect.

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Identification of 20 β-Hydroxyhydrocortisone in Rat Plasma After Administration of Hydrocortisone. (21818)

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After intravenous administration of hydrocortisone-4-C^{14}, 4 compounds more polar than hydrocortisone were detected in rat plasma by paper chromatographic methods, combined with radioautography. Four 350 g male Sprague-Dawley rats were anesthetized with sodium pentobarbital and injected via the external jugular vein with 50 μg of hydrocortisone-4-C^{14} (specific activity = 1.467 mc/m. mole) dissolved in 0.25 ml 20% ethanol in normal saline. Blood was obtained by cardiac puncture from rats sacrificed at 1, 7, 15, and 30 minutes following the injection of hormone. Plasma separated by centrifugation was extracted 3 times with chloroform, and the combined extracts taken to dryness. The residue was redissolved in 2 ml chloroform and chromatographed on a 70 x 8 mm Florisil column, using 7 ml 25% methanol in chloroform as the eluting solvent. The eluate was taken to dryness, redissolved in 0.1 ml absolute ethanol, and chromatographed on Whatman No. 1 paper for 4 days, using the benzene-formamide system of Burton, Zaffaroni and Keutmann(1).

When radioautograms were developed after 3 weeks' exposure, radioactive material was detected moving at the same rate as hydrocortisone, and this decreased progressively in amount during the observation period (Fig. 1). A more polar material (Compound 1: R, relative to hydrocortisone = 0.31, was also detected in considerable quantities, and persisted in the plasma while the radioactivity in the hydrocortisone region was disappearing. Hydrocortisone-4-C^{14} purified by paper chromatography was also added to chloroform and subjected to the same procedure as the plasma extracts. Radioautography showed no evidence of conversion to Compound 1. Despite prolonged rechromatography in chloroform-formamide, Compound 1 could not be resolved into more than one component. Small quantities of a still more polar material (Compound 2: R, relative to hydrocortisone = 0.16) were also found. When radioautograms were developed after 5 weeks' exposure, faint traces of 2 more compounds were detected between Compound 1 and hydrocortisone. Their R values relative to hydrocortisone were 0.73 (Compound 3) and 0.51 (Compound 4).

Chromatography of the original hydrocortisone-4-C^{14} showed minute traces of 4 radioactive impurities, of which 2 moved near the solvent front in the benzene-formamide system, and 2 moved more slowly (R, relative to hydrocortisone = 0.29 and 1.82).

Larger amounts of Compound 1 were prepared by extraction and chromatography of plasma obtained 10 minutes after intravenous injection of 10 mg of non-radioactive hydrocortisone per rat. The region corresponding to Compound 1 on the chromatogram exhibited ultra-violet absorption (254 mμ source), failed to reduce blue tetrazolium, and gave a yellow fluorescence with NaOH(2). The material moved at the same rate as an authentic sample of Δ^4-pregnene-11β, 17α, 20β, 21-tetrol-3-one monohydrate (20β-hydroxyhydrocortisone; Reichstein's Compound E) in the benzene-formamide, chloroform-formamide and toluene-75% methanol systems, and a mixed chromatogram in chloroform-formamide gave a single spot. On elution from the paper, a material was obtained which showed an ultra-violet absorption peak at 242 mμ in ethanol. The absorption spectrum in sulphuric acid, determined after purification by rechromatography in toluene-75% methanol, was similar to that of the 20-hydroxylated derivatives of hydrocortisone; but the presence of a peak at 470 mμ at 2 hours was more suggestive of the 20β-epimer(3). On acetylation a compound was obtained which moved at the

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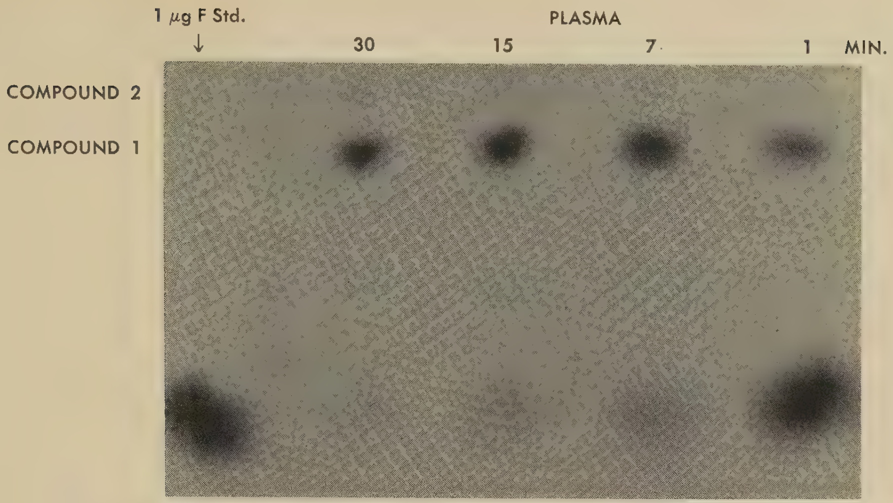

FIG. 1. Radioautogram of rat plasma extracts after intravenous administration of 50 μg hydrocortisone-4-C^{14}.

same rate as an authentic sample of Reichstein's E diacetate in benzene-formamide. The infra-red spectrum of the acetylated compound was identical with that of Reichstein's E diacetate. The eluate from the Compound 1 region was also estimated for Δ^4-3-ketosteroid content in terms of Reichstein's E using alkaline fluorometry(4), and subsequently assayed for carbohydrate activity using the mouse liver glycogen test(5). The material was found to have carbohydrate activity which was less than that of hydrocortisone, and of the same order as the authentic Reichstein's E (Table I).

After evisceration according to the method of Ingle(6), the amounts of Compound 1 detected in plasma following hydrocortisone-4-C^{14} administration were greatly reduced.

TABLE I. Effect of Various Steroids upon Liver Glycogen Deposition in Adrenalectomized Mice.

Steroid	Dose, μg	No. of mice	Liver glycogen, mg/100 g mouse
Compound 1	28	7*	3.9 ± .97‡
Reichstein's Compound E monohydrate	30	7*	4.3 ± 1.2
Hydrocortisone alcohol	15	7*	12.7 ± 3.3
Controls	—	7*	1.0 ± .4
Reichstein's Compound E monohydrate	500	7†	41.2 ± 7.9
Controls	—	8†	2.8 ± .5

* Rockland Farms all-purpose albino mice.
† " " Swiss-Webster albino mice.
‡ Mean ± stand. error.

Summary. (1) After intravenous administration to rats, hydrocortisone-4-C^{14} was rapidly converted into several more polar compounds, detected by paper chromatographic methods combined with radioautography. (2) Evidence suggests that the most abundant of these is 20β-hydroxyhydrocortisone (Reichstein's Compound E). (3) An authentic specimen of this compound was found to be glycogenic in adrenalectomized mice but to be less active than hydrocortisone. The authentic compound corresponded in activity with the material isolated from rat blood.

We wish to thank the Endocrinology Study Section of the U. S. Public Health Service for generous supplies of hydrocortisone-4-C^{14}; Dr. S. Bernstein of the American Cyanamid Co. and Dr. J. J. Schneider of Jefferson Medical College for generous supplies of Reichstein's Compound E diacetate and monohydrate respectively; and Dr. S. Lieberman of Columbia University College of Physicians and Surgeons for the measurement of the infra-red spectra.

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Antituberculosis Activity of Substituted Thioureas. IV. Studies with 4-butoxy-4′-dimethylaminothiocarbanilide (Su 1906). (21819)

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In previous publications(1-3), it was reported that certain disubstituted thiocarbanilides exerted appreciable *in vitro* and *in vivo* activities against the human tubercle bacillus. Among the many derivatives studied, 2 were of particular interest because of their low toxicity and high activity in experimentally infected animals; namely, 4-ethoxy-4′-isobutoxy-thiocarbanilide (Su 1795) and 4-butoxy-4′-dimethylaminothiocarbanilide (Su 1906). Buu-Hoï and his collaborators(4,5) have confirmed our results, including in their studies other derivatives belonging to this class of compounds. Preliminary reports on the clinical evaluation of the 2 above-mentioned substances have been presented by Schwartz (6,7).

In the course of our studies(1), we observed that *in vitro* resistance of the H37Rv strain of *Myco. tuberculosis* toward the 2 aforementioned thiocarbanilides develops slowly. This was of interest in view of the fact that tubercle bacilli rapidly develop *in vitro* resistance to streptomycin and isoniazid (8), paralleling the early emergence of resistant strains in patients being treated with these drugs(9-11). We have accordingly extended our earlier *in vitro* work by 1) compar-

TABLE I. Rates of Development of *In Vitro* Resistance to Su 1906, Streptomycin, and Isoniazid.

	Minimal inhibiting concentrations (γ/ml)					
	H37Rv strain				BCG strain	
No. of transfers	Su 1906	Streptomycin 1st series	Streptomycin 2nd series	Isoniazid	Su 1906	Isoniazid
1	3.9	1.0	2.0	.04	2.0	.02
2	3.9	7.8	7.8	2.5	3.9	.04
3	7.8	7.8	62.4	124.8	15.6	.7
4	7.8	7.8	62.4	249.6	15.6	124.8
5	3.9	7.8	124.8	124.8	15.6	124.8
6	62.4	62.4	>3000.0	124.8	31.2	249.6
7	62.4	62.4		124.8	31.2	
8	15.6	>128.8		124.8	31.2	
9	62.4			249.6	31.2	
10	62.4			249.6	31.2	
11	62.4					
12	62.4					
13	62.4					
Degree of resistance developed on:						
5th transfer	1 ×	7.8 ×	64 ×	3200 ×	8 ×	6240 ×
6th "	16 ×*	62.4 ×	1530 ×	3200 ×	16 ×	12000 ×

* Denotes that minimal inhibiting concentration on 6th successive transfer has been increased 16-fold.

TABLE II. Influence of a Sub-Inhibiting Concentration of Su 1906 (2 γ/ml) on the Emergence of H37Rv Resistance to Streptomycin or Isoniazid.

Transfer No.	Minimal inhibiting concentration of Su 1906, γ/ml	Minimal inhibiting conc. of streptomycin, γ/ml		Minimal inhibiting conc. of isoniazid, γ/ml	
		In absence of Su 1906	In combination with Su 1906	In absence of Su 1906	In combination with Su 1906
1	3.9	1.0	.5	.016	.031
2	3.9	2.0	.5	.063	.031
3	3.9	3.9	.3	.13	.063
4	15.6	7.8	.3	1.0	.063
5	15.6	7.8	.3	7.8	.25
6	15.6	15.6	1.0	30.0	.25
7	15.6	31.2	1.0	>125.0	1.0
8	15.6	31.2	1.0		
9	15.6	62.4	1.0		
10	15.6	62.4	2.0		
11	15.6		7.8		
12	15.6		3.9		
13			31.2		

ing the rates of emergence of resistant strains of H37Rv and BCG to Su 1906 with that induced by streptomycin and isoniazid, 2) determining the influence of Su 1906 upon the emergence of resistance to streptomycin or isoniazid, and 3) establishing the activity of Su 1906 against H37Rv strains previously made resistant to either streptomycin, isoniazid, Tibione, or p-aminosalicylic acid (PAS).

Methods. Cultures were grown in a modified liquid Kirchner medium containing Tween 80 and bovine serum albumin (Fraction V). For the initial assays, the inoculum was 0.1 ml per 10 ml of medium and consisting of a 1:20 dilution of a 7-day-old culture grown in this medium. For subsequent assays the inoculum was 0.1 ml of broth culture taken directly from a tube of the preceding assay and containing a sub-inhibiting concentration of the drug. All tubes were incubated for 14 days at 37°C and the minimal inhibiting concentrations then recorded. Because of the relatively low solubility of Su 1906 in water, 0.1 N HCl was used in preparing the stock solution (100 mg%) with aliquots then being added to the assay broth tubes to obtain the various concentrations.

Results. (*a*) *Rate of development of in vitro resistance.* The relative rates of development of resistance to Su 1906, streptomycin and isoniazid were studied in an experiment in which the two strains of *Mycobacterium tuberculosis,* H37Rv, and BCG, were transferred at intervals of fourteen days into freshly prepared serial dilutions of these drugs. The results of this experiment are recorded in Table I.

From the results recorded in Table I, it is evident that resistance to Su 1906 develops at a considerably slower rate than is the case with streptomycin and isoniazid. The differences in this acquired resistance are particularly marked after the 5th or 6th transfer.

(b) *Influence of Su 1906 upon the emergence of resistance to streptomycin and isoniazid.* In the following experiment we have investigated whether the development of resistance of *Mycobacterium tuberculosis* H37Rv toward streptomycin or isoniazid is retarded in the presence of a fixed (2γ/ml), but sub-inhibiting concentration, of Su 1906. The results shown in Table II indicate that the presence of 2 γ Su 1906/ml of medium appreciably delays the development of resistance to streptomycin and almost completely suppresses it in the case of isoniazid.

(c) *Sensitivity to Su 1906 of strains resistant to various antituberculosis compounds.* The cross-resistance between Su 1906 and various known anti-tuberculosis compounds was then studied. As shown in Table III, strains of *Mycobacterium tuberculosis* H37Rv resistant to streptomycin, isoniazid, or PAS were fully sensitive to the action of Su 1906. This result was not unexpected, since these four substances are chemically unrelated. On

TABLE III. *In Vitro* Activity of Su 1906 against Strains of H37Rv Made Resistant to Other Anti-Tuberculosis Agents.

		Min conc. (γ/ml) of Su 1906 capable of completely suppressing growth of organism		
Strain & extent of resistance		1st test	2nd test	3rd test
	γ			
PAS-resistant	125*	3.9	3.9	7.8
Streptomycin-resistant	125	3.9	7.8	7.8
Isoniazid- "	125	3.9	.25	1.0
Su 1906- "	62.4	31.2	31.2	62.4
Tibione- "	125	62.4	31.2	90.0

* Denotes that this strain will grow in the presence of 125 γ PAS/ml broth.

the other hand, cross-resistance was found to exist between Su 1906 and Tibione by employing a strain of *Mycobacterium tuberculosis* H37Rv made resistant to Tibione by serial transfer. Originally, this strain was sensitive to 0.5 γ Tibione/ml of medium; but after 16 transfers, it was capable of growing in the presence of 62 γ of Tibione/ml of medium. In 3 consecutive tests, this Tibione-resistant strain demonstrated an equally high degree of resistance toward Su 1906, tolerating from 31 to 62 γ of this compound per ml of culture medium. Tibione (para-acetylaminobenzaldehyde thiosemicarbazone) is a monosubstituted thiourea, whereas Su 1906 (4-butoxy-4′ dimethylamino thiocarbanilide) is a disubstituted thiourea. This finding indicates that Tibione and Su 1906 probably act upon a similar, if not identical, biological system within the tubercle bacillus.

Although we shall report elsewhere on the *in vitro* action of Su 1906 upon various resistant strains, it is of interest to note that mice infected with a virulent, Su 1906-resistant strain of H37Rv failed to respond to treatment with normally effective doses of Tibione.

Discussion. The experiments recorded in this paper have shown that the H37Rv and BCG strains of *Myco. tuberculosis* develop resistance to the disubstituted thiourea, Su 1906, more slowly than is the case with either streptomycin or isoniazid. Since it is common experience that the development of bacterial resistance in humans toward the various anti-tuberculosis agents parallels the rate of resistance brought about by *in vitro* methods, it may be assumed that, in humans, resistance of *Myco. tuberculosis* toward Su 1906 is also developed slowly. It has not been possible thus far to adequately study this question in experimental animals. As Wolinsky and Steenken(10) have shown for streptomycin, resistant strains of mycobacteria emerge only irregularly in experimentally infected mice, guinea pigs, and rabbits treated for prolonged periods with this antibiotic.

The results of studies on the rate of emergence of resistance with combinations of Su 1906 with streptomycin or isoniazid is of practical interest. They suggest that such combinations may bring about similar effects in human therapy, and that the presence of Su 1906 retards the development of resistance to streptomycin or isoniazid.

The cross-resistance observed between Tibione and Su 1906 is of particular interest; this finding suggests that either the thiourea component of the thiosemicarbazone is the responsible factor for the anti-tuberculous activity of Tibione, or that a metabolite common to both substances is formed in the animal body which is directly involved in their anti-tubercular action.

Summary. 1. *In vitro* resistance to 4-butoxy-4′-dimethylamino thiocarbanilide (Su 1906) develops at an appreciably slower rate than that developed to streptomycin, PAS, or isoniazid. 2. A combination of a sub-inhibiting concentration of Su 1906 with either streptomycin or isoniazid, considerably retards the emergence of streptomycin or isoniazid resistant strains. 3. A state of cross-resistance exists between Tibione and Su 1906.

Acknowledgment is made to Mr. Leonard Lewis who performed a portion of the technical work described.

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Application of I^{131} to Determination of Viral Antibodies. (21820)

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Within recent years the radioactive isotope of iodine, I^{131}, has been employed by several groups of investigators as a means of labeling antisera for immunological studies *in vitro* (1-7). It has been found that when the amount of iodine introduced into the protein molecule is kept at a low level, no change can be detected in the immunological reactivity of the antibody. This technic provides a useful method of measuring sedimented antibodies when determination by nitrogen analysis is difficult or impossible. The possibility of applying the method to the demonstration and determination of viral antibodies has been explored, and the results of studies with 3 different virus-antiserum systems are presented here.

Methods. Viruses and sera. The viruses used in these studies were: the French neurotropic strain of yellow fever(8); the virus of Bwamba fever(9); the PR 8 strain of influenza. Infected mouse brain was used as the source of yellow fever and Bwamba fever viruses, the pools used having 50% lethal endpoint titers of approximately 10^{-8} and 10^{-5}, respectively, on intracerebral titration in adult mice. The source of influenza virus was infected allantoic fluid from embryonated eggs, the fluid having a hemagglutination titer of 1:2048 when titrated with 0.5% chick red blood cells. The sera used were: normal and immune human for yellow fever; normal and immune rhesus monkey for Bwamba; normal and immune ferret for influenza. *Radioiodination.* The method of serum iodination was patterned after that of Masouredis *et al.*(4) except that whole serum was used instead of a γ-globulin fraction. 100-200 μc of carrier-free I^{131}† in 0.025-0.04 ml 0.1 N I_2-0.1 M KI were added per ml of serum. Following iodination, the sera were dialyzed against 18 liters of phosphate buffer, pH 7.6. After dialysis, 95-98% of the radioactivity remaining was precipitable with trichloroacetic acid. Prior to mixture with virus or control preparations, all dialyzed sera were centrifuged at the speed and for the time period to be used to sediment the pellet in the final stage of the experiment. The supernatant fluid from this preliminary centrifugation was used in the test. Samples of serum were removed before and after dialysis for assay of radioactivity and, after dialysis, for protein nitrogen determination. Protein nitrogen was determined colorimetrically with Nessler's reagent after acid digestion of the samples. For measurement of radioactivity, the serum samples were diluted in buffer as necessary, plated in small stainless

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TABLE I. Radioactivity and Calculated Serum Protein Nitrogen of Sediments from Serum-Virus and Serum-Control Mixtures.

			Serum protein-bound I^{131} counts/sec.		Final sediments	
Virus	Preparation	Serum	Per ml serum	Per mg serum protein N	Total counts/sec.	μg serum protein N (calculated)
Yellow fever	N*	N	14,576	1422	25	18
	V	N	14,576	1422	19	13
	N	I	10,303	1099	9	8
	V	I	10,303	1099	9	8
Bwamba fever	N	N	20,740	2700	319	118
	V	N	20,740	2700	435	161
	N	I	19,260	1910	295	155
	V	I	19,260	1910	365	191
Influenza	N	N	3610	520	33	64
	V	N	3610	520	55	105
	N	I	3430	587	35	59
	V	I	3430	587	80	137

* N = Normal; V = Virus infected; I = Immune.

steel cups with the aid of a detergent-spreading agent, and dried under a heating lamp. The pellets obtained from the serum-virus and serum-control mixtures were dissolved in a few drops of dilute NaOH and similarly plated. Counting was done with an end-window Geiger Counter tube and a Tracerlab Autoscaler. The efficiency of the system, as determined with National Bureau of Standards I^{131}, was 12.5%

Results. Preliminary experiments using the yellow fever virus system showed that no adverse effect upon viral infectivity resulted from exposure of virus suspensions for periods of several hours to dialyzed normal serum which had been iodinated with radioiodine. In addition, no decrease in the effectiveness of neutralization was observed to result from radioiodination of immune serum.

In Table I are presented the results of experiments carried out with the three virus systems. For the yellow fever virus study, 10% normal and infected mouse brain suspensions were treated with protamine sulfate followed by heparin according to the method of Warren(10). This technic precipitates much of the extraneous brain material without removing a detectable amount of this strain of virus (11). The partially cleaned preparations were further purified by sedimentation in an ultracentrifuge, and the sediments resuspended to ½ the original volume in 0.75% bovine albumin in buffered saline. Three ml portions were mixed with 2.5 ml portions of normal or immune sera which had been radioiodinated. The mixtures were centrifuged for three hours at 27,000 rpm, and the pellets washed by suspension in bovine albumin solution and resedimentation.

For the Bwamba fever virus study, 10% normal and infected mouse brain suspensions were treated by low speed centrifugation followed by passage of the supernatant fluid through columns of the cation exchange resin, Amberlite XE (IRC 50).‡ The pH and ionic concentration of the resin columns were so adjusted as to permit the virus to pass through the column while removing a considerable part of the tissue material which would sediment with the virus on centrifugation(11). The resin-treated fluids were then concentrated by sedimentation in the ultracentrifuge and resuspension to 1/20 the original volume in bovine albumin solution. 0.8 ml portions were then mixed with 1.0 ml of radioiodinated normal or immune monkey serum. The mixtures were incubated at 5°C overnight and then centrifuged for one hour at 10,000 rpm. The sediments were washed 3 times by suspension in bovine albumin solution and resedimentation.

The influenza virus study employed normal and infected allantoic fluids concentrated by sedimentation at 13,000 rpm. The sediments were washed once, resedimented, and suspended to 1/100 their original volume in buffered saline. 0.8 ml portions of the concentrates were mixed with 0.8 ml portions of radioiodinated normal or immune ferret sera. The mixtures were incubated overnight at 5°C, following which they were centrifuged for 1 hour at 13,000 rpm, and the pellets washed twice by suspension in saline and recentrifugation.

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‡ Rohm and Haas Co., Philadelphia.

The data presented in the Table are given as the counts per second observed per sample and, for the sediments, as the μg of sedimented serum nitrogen calculated from the specific radioactivity of the serum proteins. The values of sedimented serum nitrogen were arrived at by assuming an equal distribution of bound I^{131} among all available proteins. It is recognized that this assumption may or may not be valid, but it has been utilized since it provides the only means of comparing data obtained with sera having significantly different amounts of bound radioactivity.

The pattern of the results obtained in the yellow fever and Bwamba fever virus studies differed, but it could not be shown that a detectable portion of the pellet from the virus-immune serum mixtures could be attributed to specific antibody. In the Bwamba virus experiment, the combination of infected brain and immune serum produced the highest value, in terms of sedimented serum nitrogen. However, this appears to be due to a combination of effects: (1) the infected brain gave higher values with either serum than did normal brain; and (2) the immune serum gave higher values with either brain preparation than did the normal serum. Of significance to this study is the fact that the use of more concentrated and less purified tissue suspensions, in the Bwamba virus study, led to greatly increased nonspecific values in the final pellets. Trials with the γ-globulin fraction of sera, prepared by a slight modification of the method of Jager and Nickerson(12), did not suggest any improvement over the use of whole serum for either the yellow fever or Bwamba fever virus systems.

In contrast to the first two studies, the results with the influenza system suggest the presence of a specific immunological component in the final pellet derived from the mixture of virus and immune serum. As was the case in the Bwamba virus experiment, the degree of nonspecific sedimentation was high, this being more marked in the preparations containing virus. But unlike the preceding experiment, when the difference between the μgs of serum nitrogen sedimented in the presence or absence of virus is compared for each serum, 37 μgs of serum nitrogen presumably may be attributed to sedimented antibody. That it is not an artefact related to differences in nonspecific sediments derived from the two sera is suggested by the essential agreement of the values obtained from the 2 sera when mixed with the normal allantoic fluid concentrate.

Discussion. Although the use of radioiodinated antisera does not appear to lend itself easily to the determination of viral antibodies, the results with the influenza system suggest that it may be of potential value under the proper conditions. One obvious difficulty encountered was the excessive amount of nonspecific serum protein sedimented when fairly crude concentrated control and virus suspensions were used. This was sufficient to have obscured specific antibody if present in small amount. It is possible that the nonspecific components might be reduced by saturation of the reagents with unlabeled protein after the technic of Boursnell *et al.*(7). It may be significant that the only apparently positive results obtained utilized the most highly concentrated antigen preparation.

It would be of great interest to know whether differences in the mechanism of virus-antibody interaction contributed to the failure or success of antibody demonstration. The use of labeled antisera might provide an effective method of studying such mechanisms. To explore this problem it would seem necessary to obtain virus preparations which are both sufficiently purified to reduce nonspecific sedimentable activity to a low level, and sufficiently concentrated so that the amount of viral antigen used could be varied over a considerable range. The use of γ-globulin rather than whole serum might also be advantageous, but our experience suggests that the nature of the virus preparations was the major complicating factor.

Summary. Experiments are described in

which preparations of yellow fever virus, Bwamba fever virus and influenza virus were allowed to react with corresponding antisera labeled with radioactive iodine (I^{131}). No significant differences were observed in the activity of the sediments obtained with the first 2 virus systems from those of appropriate controls. The influenza virus-immune serum sediment showed significantly more activity than any of its control preparations. It is suggested that the technic may be of value in virus studies where relatively pure and concentrated virus preparations are available.

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Comparison of Acetylcholinesterase Activity in Intact and Homogenized Nerve. (21821)

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In studies on the squid giant axon, Rothenberg, Sprinson, and Nachmansohn(1) demonstrated that quaternary ammonium compounds like acetylcholine were unable to reach the axoplasm from the solution bathing the fiber, whereas tertiary ammonium compounds like trimethyl amine could readily penetrate the membrane and hence be recovered from the axoplasm. Using this differential behavior as a basis for study, Wilson and Cohen(2) have recently distinguished an "external" and "internal" acetylcholinesterase in the peripheral nerves of the spider crab, *Libinia emarginata* (Rathbun), by comparing the hydrolytic rates of acetylcholine chloride (AchCl) and the tertiary amine, dimethylaminoethyl acetate (DMAEA) in the same intact nerves after treating with inhibitors, blocking agents, and x-ray irradiation. Under the optimal conditions, *i.e.* where the DMAEA could reach the most "internal" enzyme, Wilson and Cohen showed that about 75% of the total acetylcholine-esterase is found "internal" to the barrier for quaternary ammonium compounds. Earlier reports(3,4) have shown that from 25% to 60% of the total enzyme is "external" to this barrier.

In the present paper a comparison has been made between intact spider crab nerves and the homogenate prepared from the same nerve in their ability to hydrolyze acetylcholine. The difference between these values is referred to here as the "internal" acetylcholinesterase. The term should be distinguished from the same one used by Wilson and Cohen(1) who define it according to their experimental procedure as the "additional enzyme which can hydrolyze DMAEA" in the intact nerve. Both cases of course refer to the enzyme which lies within the physico-chemical barrier to quaternary ammonium compounds.

Methods. The nerves used were from the meropodite of the claw and walking legs of

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[*] This work was completed while author was a Fellow of the Grass Trust for Research in Neurophysiology.

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TABLE I. Summary of Data on Acetylcholinesterase Activity in Intact and Homogenized Nerves of the Spider Crab.

1 Nerve No.	2 Fresh wt, mg	3 Intact nerve Q_{ChE}, mg/hr/g	4 Homogenized nerve Q_{ChE}, mg/hr/g	5 "Internal" Q_{ChE}, mg/hr/g	6 % "Internal" Q_{ChE}
1	10.0	29.0	49.0	20.0	40.8
2	11.8	38.1	66.9	28.8	43.0
3	12.2	37.2	75.8	38.6	50.9
4	23.6	37.1	52.1	15.0	28.7
5	29.2	31.1	44.8	13.7	30.5
6	49.4	15.1	34.6	19.5	56.3

the spider crab *Libinia emarginata* (Rathbun). Dissected nerves 5-8 cm long were tested and found to be electrically active before chemical analysis. After blotting and weighing, the nerves were placed in 3.3 ml of a buffered solution (pH 7.5) of the following composition: 0.1 M NaCl; 0.05 M $K_2HPO_4 \cdot 3 H_2O$; 0.2 M KCl. The temperature was maintained below 15°C until 10-15 minutes before the hydrolysis reaction was started. A stock solution of acetylcholine chloride, prepared for each experiment, was of such concentration that when 1.0 ml of this solution was added to the 3.3 ml of buffer, the final concentration of AchCl was 3.2 μM/ml. The intact nerve, buffer, and substrate were shaken together mechanically at room temperature (about 25°C) for one hour. At 0, 20, 40, and 60 minutes after the addition of the substrate, 1.0 ml samples of the remaining solution were removed and tested colorimetrically for the residual substrate by the hydroxamic method(5). At the end of the hour the nerve was washed in fresh, cold buffer for 45 minutes to 2 hours, and ground in a glass tissue homogenizer containing 0.5 ml buffer and small amounts of quartz powder. The grinding was carried out in an ice water bath and generally was complete in 2-3 minutes. The resulting mixture was brought up to volume with the buffer, and the AchCl added to provide the same substrate concentration as before. Samples for analysis were removed at 20, 35, 50 and 65 minutes after the addition of the substrate and tested as before.

Results. The results are summarized in Table I for six nerves. Column 2 shows the fresh weight of each nerve, placed in the order of increasing weight from 10.0 mg to 49.4 mg.

Columns 3 and 4 show the activity of acetylcholinesterase (Q_{ChE}) in mg per hour per g of fresh tissue for intact and homogenized nerves respectively. The values are based on an average of at least 2 consecutive periods during which the hydrolytic rate was practically constant. Values range from 15.1 to 38.1 mg/hr/g for the intact nerve, and 34.6 to 75.8 mg/hr/g for the homogenized nerve.

In column 5 the "internal" Q_{ChE} was calculated by subtracting the "external" Q_{ChE} (as determined in the intact nerve) from the total Q_{ChE} (the homogenate). This assumes of course that the Ach barrier is almost or completely destroyed during the homogenizing process. Homogenizing longer than 2-3 minutes did not increase the enzyme activity, but rather tended to lower it. The values therefore represent a maximum by the present methods and range from 13.7 to 38.6 mg/hr/g (average: 22.6 mg/hr/g).

The last column shows the percentage of "internal" Q_{ChE} which ranges from 28.7% to 56.3% (average: 41.7%). The standard deviation is ± 10.1.

Summary. The data indicate therefore that somewhat more than 50% of the total acetylcholinesterase in crab nerve lies outside the barrier to quaternary ammonium compounds. The role of this large quantity of enzyme remains obscure at present since Wilson and Cohen(1) have shown that inhibition of the "external" enzyme does not prevent nervous conduction.

The author wishes to thank Drs. D. Nachmansohn and I. B. Wilson for their helpful advice during this short study.

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Reduction by Glucagon of Glycogen Deposition Effect of Insulin in Rat Diaphragm.* (21822)

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Gemmill(1) showed that the synthesis of glycogen by the isolated rat diaphragm in a buffered medium containing glucose was increased when insulin was added. Stadie, *et al.*(2) have used the term "insulin effect" in referring to this phenomenon. R.-Candela and co-workers(3-6) found that the insulin effect was greatly reduced when glucagon (HGF) was also added to the medium. This was demonstrated by determinations of glycogen in the muscle and the uptake of glucose from the incubation medium. This is a preliminary report of work which confirms R.-Candela's findings. An incubation medium was used which contained a small amount of labeled (C^{14}) glucose in addition to 500 mg% glucose. A C^{14} determination made on the glycogen isolated from the muscle at the end of the incubation time gave an estimate of the glycogen synthesis during the test period. Chemical determinations were made of total glycogen of the muscle and of the glucose uptake from the medium. The determined values for the C^{14} label in diaphragm glycogen show good agreement with values for total glycogen and for glucose utilization.

Methods. Rat hemidiaphragms were incubated in Warburg flasks in the presence of glucose, labeled glucose, and, except for the controls, insulin or insulin plus glucagon. The general methods outlined by Stadie *et al.*(2) were followed in the incubation procedure. In addition to phosphate buffer containing 500 mg% glucose, each flask contained enough labeled glucose to give approximately 100,000 counts per ml/min. Insulin (Lilly 208-108B-50)‡ was present in a concentration of 0.5 unit per ml and glucagon (Lilly 208-158B-197) when present, was in a concentration of 0.1 mg/ml. At the conclusion of the incubation period the muscle was removed, washed for one minute in cold buffer containing 500 mg% glucose but no labeled glucose, placed in 3 ml hot KOH, and the glycogen subsequently precipitated with alcohol. Total glycogen values were determined using anthrone. An aliquot of the glycogen was further refined and plated for C^{14} determination. Oxygen uptake was measured to insure that each hemidiaphragm was respiring during the 90-minute incubation period. C^{14} in the CO_2 taken up in NaOH during the incubation was measured. The residual glucose was measured according to the micromethod of King(7) modified for a sample of 0.1 ml.

Results. The addition of glucagon invariably resulted in a smaller "insulin effect" than when insulin alone was present. The differences shown in Fig. 1 are averages of the differences between the 2 half diaphragms of single rats. The differences for each kind of determination were compared statistically and *t* values computed. The resulting P values appear in Fig. 1. Fisher(8) describes a test

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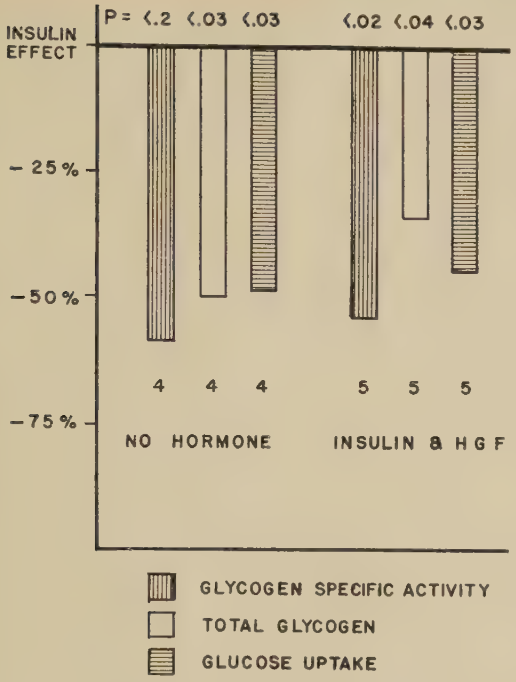

FIG. 1. Comparison of effects of incubation of rat hemidiaphragm in presence of insulin with corresponding tissue in presence of either no hormone or of insulin plus glucagon. Upper horizontal line indicates insulin effect on hemidiaphragms. Vertical bars indicate differences between insulin effect and effect of designated treatment. Numbers below bars show number of experiment. P values are based on actual differences, not percentages.

utilizing χ^2 for combining the P values of a number of kinds of determinations related to the same phenomenon to give a single P value indicating the likelihood of the series of determinations occurring by chance. This test gave a P value of $<.02$ for the control experiments and a P value of $<.01$ for the experiments comparing insulin and insulin plus glucagon.

Discussion. Although glucagon has no effect upon muscle glycogen when insulin is absent, it definitely has an effect in the presence of insulin. The results so far obtained do not give any indication as to the mechanism of this action. It probably is not correct to speak of an anti-insulin action, since there is no evidence of an inhibition of insulin itself, but there is certainly a reduction of the insulin effect.

The amount of glucagon used to demonstrate this inhibition was relatively large: about four times the insulin by weight of the relatively pure amorphous products used. The suppression of the insulin effect was, however, not complete, that is, the values for insulin plus glucagon exceeded the control (no insulin or glucagon).

Previous *in vivo* work with the depancreatized dog in this laboratory(9) showed that when C^{14} labeled glucose was given, the specific activity of muscle glycogen was significantly higher in animals given insulin alone as compared with those given insulin plus glucagon. Glucagon alone had no effect.

Further *in vitro* work is being done with rat diaphragm to determine the glucagon influence at different insulin and glucagon concentrations, and at different glucose concentrations.

Summary. Increase in glycogen content of rat diaphragm incubated in the presence of glucose and insulin is considerably smaller when glucagon as well as insulin is present.

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Comparative Behavior of I^{131} and C^{14} Labelled Albumin in Plasma of Man.* (21823)

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Proteins labelled by I^{131} trace iodination have been used to study: traumatic shock(1); turnover of albumin in normal subjects(2,3) and in Laennec's cirrhosis(4,5); behavior of I^{131} labelled insulin and corticotropin(6,7); and in gastrointestinal physiology(8,9). The physiological validity of studies based on I^{131} labelled proteins, however, has been accepted with reservations since this labelling technic introduces a foreign group into the protein. The suspicion that the I^{131} labelled protein is significantly altered is supported by the finding that the plasma disappearance rate of biosynthetically labelled N^{15} albumin(10) and S^{35} albumin(11) is considerably slower than that reported for I^{131} albumin(2,3). In addition, when internally labelled S^{35} albumin is compared simultaneously with I^{131} albumin in the same subject, the S^{35} albumin has a half time 2 to 5 times greater than the I^{131} albumin(12). This difference between native and I^{131} proteins cannot be accounted for by early non-metabolic loss of the I^{131} label, since the evidence available indicates that the I^{131} label is firmly bound to the protein during the intravascular lifetime of the I^{131} labelled protein(13,14). The possibility that I^{131} proteins metabolically behave differently from native proteins assumes further interest in view of the fact that immunochemically such proteins are indistinguishable from native proteins(15-17).

In the present study the problem of metabolic specificity of I^{131} labelled proteins was investigated by comparing the behavior of I^{131} albumin with biosynthetically labelled C^{14} albumin in 2 patients.

Materials and methods. The patients, one with polycythemia vera (F.J., age 68) and the other with inactive rheumatic heart disease in mild congestive failure (E.Z., age 56), received 100 μc of glycine 2-C^{14} intravenously and 20 to 30 days later about 100 μc of I^{131} human serum albumin‡ intravenously. The patients were ambulatory and serial sampling of the plasma for C^{14} and I^{131} albumin was carried out for 60 days. *Isolation of albumin.* Albumin was isolated from the plasma by ammonium sulfate precipitation at 4° C. The plasma was diluted 10 times with distilled water and brought to a concentration of 55% with ammonium sulfate to precipitate the globulins. After standing overnight the precipitated globulins were removed by filtration, and the filtrate was saturated with ammonium sulfate and again allowed to stand overnight to precipitate albumin. The precipitated albumin was recovered by filtration and redissolved in distilled water to the original volume. The albumin precipitation as well as the preliminary precipitation and removal of globulins was carried out a total of 3 times. The final albumin precipitate was dissolved in a small volume of distilled water, cleared by filtration, and then analyzed for C^{14}, I^{131} and nitrogen. The C^{14} content of an aliquot of the isolated albumin fraction was combusted to CO_2 by the method of Van Slyke and Folch(18) and then precipitated as $BaCO_3$. The $BaCO_3$ was then reconverted to CO_2 and the C^{14} determined with a vibrating reed electrometer using a 100 ml ionization chamber and recording potentiometer(19, 23). The C^{14} activity was expressed as relative specific activity, disintegrations per minute per mg $BaCO_3$. The I^{131} content of the albumin fraction was determined with a well-type scintillation counter after dialysis against running tap water until free of ammonia. The

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† Public Health Service Research Fellow of the National Cancer Institute.

‡ Albumin was obtained from Abbott Laboratories, N. Chicago.

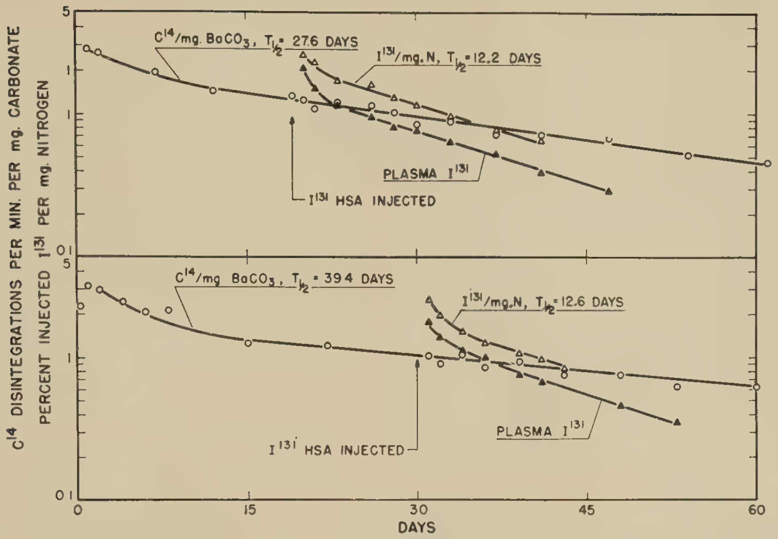

FIG. 1. Concentration-time curves for I^{131} and C^{14} albumin. Upper figure based on data derived from patient F. J., lower figure represents data obtained from patient E. Z.

nitrogen content of the I^{131} aliquot was obtained by microKjeldahl(20) and the I^{131} activity was expressed as percent injected I^{131} per mg albumin nitrogen. The albumin fraction obtained with this technic consisted of 85% albumin and 15% α and β globulin contaminants as determined by electrophoresis.

Results. Fig. 1 presents C^{14} activity, as disintegrations per minute per mg $BaCO_3$, and percent injected I^{131} per mg albumin nitrogen plotted against time for both patients. Total plasma I^{131} is also included in this figure.

Following the administration of glycine 2-C^{14} there is rapid incorporation of C^{14} into the albumin, the maximum concentration of C^{14} being achieved within 24 hours. The C^{14} activity of the albumin then decreases for the next 15 days to be followed by a slow exponential loss during the remaining 45 days of the study. Graphical analysis of the C^{14} albumin curves reveals at least 2 plasma disappearance components, a slow exponential rate with a half-time of 28 days in one patient and 39 days in the other, and a more rapid component of about 3 to 4 days.

Both the percent of injected I^{131} per mg albumin nitrogen and the total plasma I^{131} curves consist of 2 readily apparent exponential plasma disappearance rates without the build-up phase found in the C^{14} albumin curve. The slow exponential I^{131} albumin component has a half-time of about 12 to 13 days and the initial rapid exponential rate of loss occurs with a half-time of about 1 day.

The half-time values for all these components obtained from the slopes of the regression lines as well as the standard error of these slopes(25) are summarized in Table I.

Discussion. These results indicate that I^{131} albumin leaves the plasma at a rate that is more than twice as fast as the C^{14} labelled albumin. The initial rapid rate of loss of both C^{14} and I^{131} albumin from the plasma probably represents extravascular dilution (3,21). The apparent slower rate of extravascular equilibration of C^{14} albumin, which

TABLE I. Plasma Disappearance Rates of I^{131} and C^{14} Albumin.

Plasma component	Plasma half-time in days Patient F. J.	Patient E. Z.
Rapid C^{14} albumin	3.2	3.7
Slow " "	27.6 ± 1.8*	39.4 ± 3.8*
Rapid I^{131} "	1.1	1.0
Slow " "	12.2 ± 1.4*	12.6 ± 1.6*
Total plasma I^{131} slow component	12.0 ± .33*	11.2 ± .54*

* Values obtained by least squares and from the stand. error of the regression line slope(25). Other values obtained by inspection and graphical analysis.

occurs between the 2nd and 16th day after administration of the glycine 2-C^{14} is probably due to significant resynthesis of C^{14} albumin during this period. The equilibration portion of the C^{14} albumin curve, therefore, represents the summation of two competing effects, loss of C^{14} albumin from the plasma as a result of dilution and degradation opposed by the addition of newly synthesized C^{14} albumin.

At least 3 factors must be considered in interpreting the difference in behavior between I^{131} and C^{14} albumin after equilibration in the extravascular space has occurred. The longer half-time of the C^{14} albumin may reflect reincorporation of degraded C^{14} into albumin being resynthesized during the terminal 45 days period of the study. The magnitude of this effect cannot be evaluated with certainty since the specific activity-time data for the albumin precursor pool is not known. Reincorporation, however, is probably insufficient to account for the prolonged half-time, since Berlin and his co-workers(22,23) have shown that there is a very rapid rate of respiratory and renal excretion of C^{14} following glycine 2-C^{14} administration in man. Within 10 days about 85 to 95% of the total C^{14} is excreted by these routes, so that less than 10% of the C^{14} activity would be available for reincorporation into albumin within the 15- to 60-day period in which the C^{14} albumin data were obtained. The values for C^{14} albumin are in good agreement with the half-time of 26 to 31 days obtained following transfusion of donor-synthesized S^{35} albumin in man (24), a study carried out to minimize the effect of S^{35} reincorporation.

The longer half-time of C^{14} albumin could also be due to the presence of contaminants in the albumin fraction, if these contaminating components which constituted about 15% of the albumin fraction, had significantly longer half-lives than the albumin. The third possibility is that the introduction of I^{131} into albumin significantly alters the metabolic behavior of the albumin yielding results that differ from the biosynthetically C^{14} labelled albumin.

The data obtained in this study are inadequate to evaluate completely the contribution of each of these possible explanations to the differences found between I^{131} and C^{14} albumin. Until more definitive data are available these results indicate that metabolic studies based on I^{131} trace-iodinated proteins should be accepted with caution. The evidence available suggests that the introduction of iodine into a protein significantly alters its metabolic behavior.

Conclusions. 1) Rapid incorporation of C^{14} into circulating albumin occurs following intravenous administration of glycine 2-C^{14} in man. The maximum C^{14} activity occurs at about 24 hours, and is followed by a loss of C^{14} albumin from the plasma which consists of a rapid exponential component with a half-time of about 3 days and a slow exponential component with a half-time of 28 to 39 days. 2) I^{131} trace iodinated albumin studied concurrently in the same patients leaves the plasma after extravascular equilibration at a rate that is more than twice as fast as the biosynthetically C^{14} labelled albumin. (Half-time of 12 to 13 days.) 3) Difference in behavior between I^{131} and C^{14} albumin suggests that introduction of I^{131} into albumin significantly alters the metabolic behavior of the albumin.

The authors gratefully acknowledge the technical assistance of Carol Hewitt and Carol Wenzel.

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Photometric Determination of Lysozyme Activity.*† (21824)

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Measurement of bacteriolytic activity has been the subject of several investigations. Fleming(1) reported the original method for lysozyme wherein time was measured as that necessary for a given portion of a material rich in enzyme, such as a drop of tears, to dissolve part or all of a full-grown plate culture of *Micrococcus lysodiekticus*. One of the first optical methods of assay was developed by Boasson(2). In this method the decrease in density from the time the enzyme was added to a bacterial suspension to a given time (60 minutes or more) was observed. The error in measurement was estimated to be 10%. Alderton, *et al.*(3,4) used a method similar to that of Boasson with a reliability of approximately 15%. Smolelis and Hartsell(5) devised a photometric method utilizing the differences in light transmission with crystalline lysozyme dilutions compared to the values for substances being tested after 20 minutes incubation at room temperature. Fraenkel-Conrat(6) has employed the method of Alderton, *et al.*(3,4) and measured the turbidimetric rate of lysis of a *Micrococcus lysodiekticus* suspension over a 3-minute period at intervals of 30 seconds. He found that at least 5 out of 6 readings agreed within 10 to 15%. The assay to be described in this paper has advantages of extreme rapidity in duplicating measurements and a good degree of accuracy.

Methods and material. The Pfaltz and Bauer Fluorophotometer (used as a filter photometer) has been employed with a sensitive multiple mirror galvanometer (direct reading; 1% transmission = 2 mm on the scale). The filter is 645 mμ (± 15 mμ). Standard 16 x 150 mm test tubes may be used for the reaction cell. A 60-second stopwatch divided into 0.2 of a second is used as a timer. Cells of *Micrococcus lysodiekticus* for inoculation were prepared from a nutrient agar slant culture No. 19 of the Waksman Collection[‡] either by growth on a solid medium (0.5% Bacto-peptone, 0.5% NaCl, 0.1% yeast ex-

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[‡] Kindly supplied by Dr. R. E. Gordon, Institute of Microbiology, Rutgers University.

tract and 2% agar) at 37°C for 48 hours, or in a liquid medium (0.5% Bacto-peptone, 0.5% NaCl, 0.3% beef extract and 0.1% yeast extract) at 30°C for 48 to 72 hours on a mechanical shaker in 250 cc Erlenmeyer flasks. The latter method produced vast quantities of cells. A detailed account of the production of these cells in submerged culture and relationships between nutrition, growth and susceptibility of the resulting cells to lysozyme will appear later.§ Cells were prepared by washing once with about 60 times the cell volume with distilled water followed by centrifugation at 20°C and 3000 rpm. This process was repeated with centrifugation at 0°C using 3 washings with cold acetone and 2 washings with cold ether. Final drying was accomplished by air and suction on sinter glass funnels. The product appeared as a fine yellow powder and may be stored in a desiccator at 5°C or in the freezer. Cells dried in this way were easily resuspended and were very susceptible to lysis by lysozyme. Contrary to the observations of Smolelis and Hartsell(7) acetone treatment did not alter desirability of the preparation although these authors had pretreated cells with phenol. Twenty-five mg of dried cells from either method of culture were suspended in 90 cc M/15 pH 6.2 phosphate buffer(6) and 10 cc 1% NaCl. Suspension of the cells was usually immediate if the organisms had been properly prepared. Five cc of the bacterial suspension and 0.5 cc of the enzyme solution were used as the assay system. The resulting suspension usually showed a 45-55% transmittance when water was set at 100% transmission. The determination may be carried out at room temperature (ca. 25°C) in the following sequence: (a) pipet 5 cc of bacterial suspension into test cell, (b) adjust suspension to 50% transmission, (c) add 0.5 cc enzyme solution by pipet or 1 cc graduate tuberculin hyposyringe and start timer immediately, (d) mix enzyme and suspension quickly, (e) read per cent transmittance every 30 seconds for 300 seconds. When tissue extracts were made, they were excised from adult male rats of a highly inbred strain of Wistar rats from the Rutgers Colony.

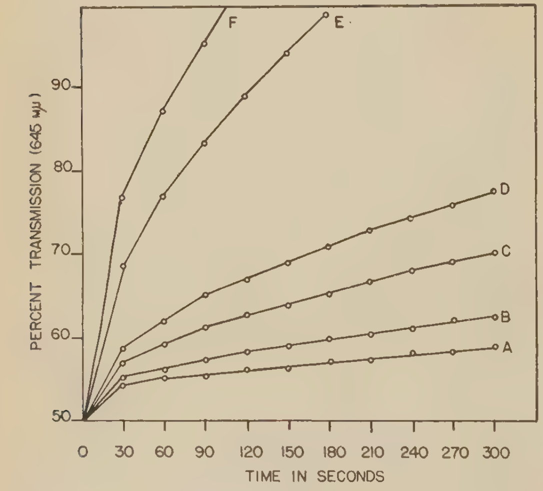

FIG. 1. Time-course activity curves with increasing concentrations of crystalline lysozyme: A = 2 μg; B = 5 μg; C = 10 μg; D = 20 μg; E = 100 μg and F = 200 μg.

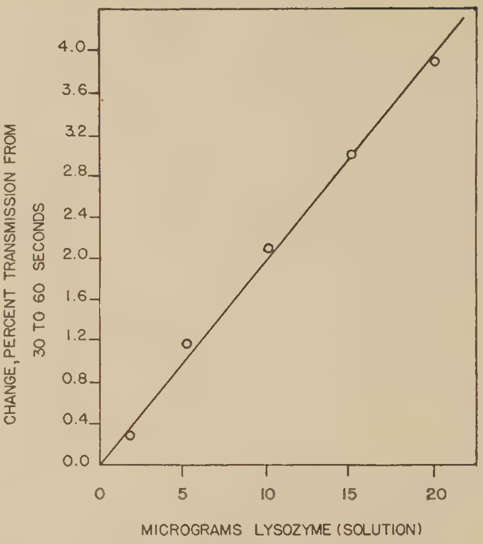

FIG. 2. Typical standard curve with crystalline lysozyme. Activity increment is from 30 to 60 sec. of a time-course curve at each concentration of enzyme.

Results. Typical activity curves for aqueous solutions of crystalline lysozyme are presented in Fig. 1. These levels of enzyme were obtained from a stock solution of crystalline egg white lysozyme at 400 μg/cc (lysozyme purchased from Worthington Biochemical Corp., Freehold, N. J.), stored at 5°C. *Activity is defined as change in per cent trans-*

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§ Litwack, G., and Pramer, D., to be published.

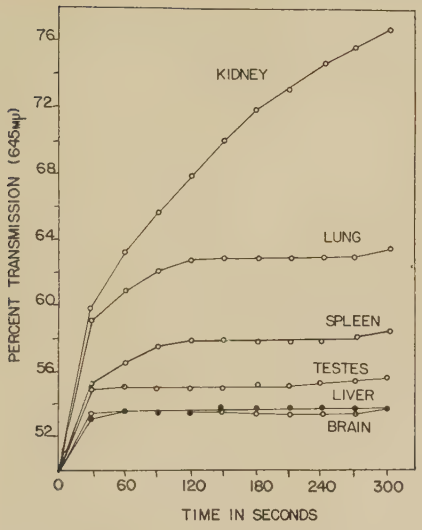

FIG. 3. Time-course activity curves from supernatants (3000 rpm; 0°C) of 1:6 water homogenates of various tissues of the rat.

mission between 30 *and* 60 *seconds of reaction.* Activity of any given sample may be easily reproduced in 60 seconds after addition of the enzyme. The changes in activity between: 0 and 30 seconds, 0 and 60 seconds, 60 and 90 seconds, 0 and 90 seconds do not yield a linear relationship under these conditions. The linear relationship between 30 and 60 seconds may, however, be consistently reproduced. A typical standard curve based on this increment of activity is shown in Fig. 2. The slight dilution effect due to addition of the enzyme solution was overcome in the first 30 seconds.

Deviations from linearity at concentrations of lysozyme above 20 μg were observed. Wilcox and Daniel(8) observed this inhibition to be partially overcome by addition of sodium ion to either enzyme or bacterial suspension. In this laboratory it has been observed that the concentration of NaCl in the culture medium also affects the susceptibility of the resulting cells to lysozyme.§

The measurements of activity are usually quickly reproducible within the range of ± 0.2% transmission which is the approximate error in reading the galvanometer scale. Reliability appears to be about 5-10%.

This rapid assay has been extended to the measurement of lytic activity extracted from mammalian blood, kidney, liver, lung and spleen as shown in Fig. 3. Kidney appears to be the most active of the tissues measured.

Summary. An accurate photometric method has been developed using a Pfalz and Bauer Fluorophotometer for measuring the lysis of suspensions of *M. lysodiekticus* by lysozyme. This method has the distinct advantages of reproducing activities in 60 seconds after addition of the enzyme. Measurement of maximal rate of lysis in the early part of the time-course of reaction decreases the possibility of interference due to synergism by proteolytic enzymes or solution of cellular material by alkali reaction. This method has been extended to extracts of mammalian tissues.

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Effect of X-Irradiation on Cecal Flora of the Rat.* (21825)

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(Introduced by William J. Darby.)

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The *in vivo* effects of ionizing radiation upon intestinal microorganisms have received increasing attention of investigators in recent years. Miller and his colleagues made exhaustive studies of the role of bacteria in post-irradiation bacteremia in mice(1,2). These studies and those of other investigators(3-5) have established a relationship between irradiation injury of the intestinal mucosa and subsequent bacterial invasion of the blood stream. Recently, Vincent, Veomett, and Riley(6) studied the post-irradiational changes in bacterial types in the small intestine of the rat beginning the third to fourth day after irradiation. None of these studies have distinguished clearly between effects of anorexia and of irradiation. The present study shows that changes in the flora are evidenced in the cecum within 24 hours after irradiation and that these changes result, at least in part, from the irradiation itself.

Methods. Male Sprague-Dawley rats weighing 300-400 g were maintained on Purina dog chow and water *ad libitum*. All rats were fasted 24 hrs prior to irradiation. Half of the rats were kept as non-irradiated controls; the other half were irradiated in groups of 3 by exposure to a single, total-body dose of 825 r (in air) delivered by a 200 KV machine, at a distance of 53 cm, with 0.5 mm Cu and 1.0 mm Al added filtration. The rate of irradiation was 36.7 r/min. The irradiated animals and pair-fed, non-irradiated controls were placed in individual metabolism cages. Water was allowed *ad libitum*, but all animals were continued on a fasting regime. The rats were anesthetized with ether 24 hrs after irradiation and the cecum was removed immediately. The contents were washed out and diluted to a definite volume with sterile physiological saline. Appropriate dilutions were plated in triplicate using standard bacteriological procedures. The following media were used to differentiate the bacterial types: Levine eosin methylene blue agar (coliforms), Rogosa's lactobacillus agar(7), and sodium azide agar. Enterococci constituted the predominant organisms growing on the azide agar, although other Gram positive organisms were also present. The plates were incubated at 37°C for 24 hrs and the colonies counted with a Quebec Colony Counter. Staining technics were employed frequently to corroborate colony identification. Dry weight determinations of the cecal suspensions permitted the calculation of the number of bacteria per gram dry weight of cecal contents.

Results. Preliminary experiments had shown that the total number of bacteria present in the pooled cecal and intestinal contents of fasted, irradiated rats was not significantly different (24 hrs after irradiation) from that of pair-fed controls. A study of the cecal

TABLE I. Cecal Bacterial Populations in Rats Subjected to 825 r Total-Body Irradiation and Non-Irradiated Control Rats.

		Counts × 10^6		
	Animals	Coliforms	Lactobacilli	Azide-resistant organisms
Exp. 1	I*	820	69	700
	C*	3	740	210
	I	1600	14	900
	C	7	900	150
" 2	I	290	340	340
	C	1	—	6
	I	240	360	600
	C	100	1600	150
" 3	I	—	19	27
	C	—	50	7
	I	98	43	200
	C	52	1100	13
	I	23	8	12
	C	3	5	20

* I = Irradiated rats sacrificed 24 hr after irradiation and 48 hr after fasting was begun. C = Non-irradiated control rats fasted 48 hr before sacrifice.

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* This paper is based on work performed under Contract AT(40-1)-1033 (No. 4) of the U. S. Atomic Energy Commission.

contents of such animals, however, revealed a definite change in predominant types. Table I shows that there was a definite decrease in lactobacilli in the cecal contents of the irradiated rats. This finding was consistent in all experiments and gains in significance when the irradiated animal is compared with its own control of the same weight sacrificed at the same time. Conversely, the coliforms and azide-resistant organisms increased in numbers in the flora of the irradiated animals. These results are similar to those obtained by Vincent *et al.*(6) in homogenate suspensions of small intestine beginning with the third or fourth day after irradiation, but more significantly at 6 to 7 days post-irradiationally. These investigators presented evidence for the interesting possibility that there normally exists a natural antagonism between lactobacilli and the other indigenous intestinal organisms and that the disappearance of this antagonism with post-irradiational decrease of lactobacilli results in increased numbers of the other types. They stated that the change could be due to the decrease in food supplied to the intestine, since irradiation ordinarily causes impaired gastric motility. Their results in the irradiated rats were compared with those in normal, non-irradiated, *non-fasted* animals. Comparison with pair-fed controls was not reported nor was mention made of the food intake of the irradiated animals. Impaired gastric motility could exert little effect, if any, in the present investigation since all rats started the post-irradiation period with essentially empty stomachs because of the previous 24-hr fast.

Discussion. Porter and Rettger(8) reported that a 5-day fast effected the type of change in intestinal microbial types observed by Vincent and coworkers(6) and by us, but they showed that a 2-day fasting period brought little discernible change. To test this basic stability in microbial proportions after 2 days of fasting, an experiment was performed in which the cecal flora of 3 rats fasted for 48 hrs was compared with that of 3 fed rats of the same age and weight. The results, shown in Table II, confirm the observations of Porter and Rettger concerning population stability after 2 days of fasting. The data show no significant change in predominant bacterial type, but do indicate a possible slight decrease in numbers of both the lactobacilli and coliforms, a result normally expected after fasting. The questionable decrease in numbers of the lactobacilli was not accompanied by the corresponding increase in either the coliforms or the azide-resistant organisms previously found in irradiated rats. Therefore, the reciprocal changes in relative numbers of lactobacilli, coliforms and azide-resistant strains in the irradiated rats are due, at least in part, to the irradiation itself and cannot be attributed solely to fasting. In view of these observations, there appears to be a need for further investigation of the effect of irradiation on intestinal and cecal microorganisms *in vivo*.

TABLE II. Cecal Bacterial Populations in Fed Rats and in Rats Fasted for 48 Hours.

	Counts × 10^6		
Treatment	Coliforms	Lactobacilli	Azide-resistant organisms
Fasted 48 hr	.55	4.5	6.8
	2.2	21	15
	.86	48	20
Fed	2.6	22	12
	9.0	110	15
	2.9	94	6.9

Summary. 1. The effect of total-body x-irradiation (825 r in air) on the cecal flora of rats has been studied. Numbers of lactobacilli, coliforms, and azide-resistant organisms in rats fasted 24 hours before and after irradiation were compared to those in pair-fed control rats fasted for the same 48-hour period. The normally predominant lactobacilli decreased in number while the coliforms and azide-resistant bacteria increased. 2. A fasting period of 48 hours elicited no proportional change in bacterial types when the counts were compared with those of normal fed rats. 3. Irradiation itself was responsible, at least in part, for the observed changes.

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Acoustic Resonance of Microorganisms to Supersonic Vibrations. (21826)

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Since the work of Wood and Loomis(1) the production and application of supersound has progressed until today it is being used as a clinical technic of physical medicine, particularly in Europe, and is receiving increasing attention in this country. Biological effects of supersound are reported in a rapidly expanding bibliography. However, few papers deal specifically with the mechanisms by which these effects are produced. Cavitation is one very apparent mechanism. Conversion of the sonic energy into heat energy is another mechanism.

Heat energy may be produced by dissipation of the energy of the supersonic wave as it travels through the conducting medium or it may be selectively produced at interfaces in the conducting medium. The above mechanisms have been explored(2). For a considerable time little else in the way of mechanism was proposed and a belief grew that heat was the only agency in producing biological effects from supersonic energy at low energy levels with cavitation appearing at very high energy levels. Ackerman(3) reported an apparent resonance of paramoecia to sonic energy at 1200 cycles per second and explained the response as resonance to a surface wave traveling to and fro down the length of the quasi-cylindrical body of the paramoecium.

Method. Using a micrometer eyepiece we have measured 100 specimens of *Paramoecium caudatum* for length, obtaining a mean of 207 μ with a standard deviation of 4.7 μ. Ludwig(4) reports the velocity of sound in various tissues of beef, man and other sources as 1490 to 1610 m/sec. (The velocity in water is 1494 m/sec.) From the above, it appears that the velocity in protoplasm is somewhat higher than in water, depending on the particular type of protoplasm. For purposes of preliminary calculation we have used the mean velocity in protoplasm as 1540 m/sec. The equation relating frequency, wavelength and velocity, is $f \lambda = v$ and the probable frequency of resonance is 3.73 mc/sec. The effective half wave length is probably shorter than the physical length of the paramoecia, (and probably the velocity in the protoplasm of paramoecia is somewhat different than in the protoplasm of beef and man). We might therefore expect the resonant frequency to be somewhat higher than 3.73 as we found to be the case. Most workers have used high power, fixed frequency, resonant transducers, and a phenomenon such as physical distortion or disruption of cell structure as the "observable." We decided to use (1) a variable frequency, (2) very low power to avoid the obscuring effect of heat and cavitation, (3) a non-resonant transducer, (4) a physiological behavior as the observable. The instrumentation employed was simple. A Hewlett-Packard oscillator, model 650A supplied the electrical oscillations the output of which was fed to a 10 mc/s Brush cylindrical ceramic crystal #C55097, (Fig. 1). It was necessary to bias this crystal in order to stabilize its activity. We were very careful to wash the crystal out with distilled water between samples to prevent contamination of the crystal and to avoid mixing of successive samples. A

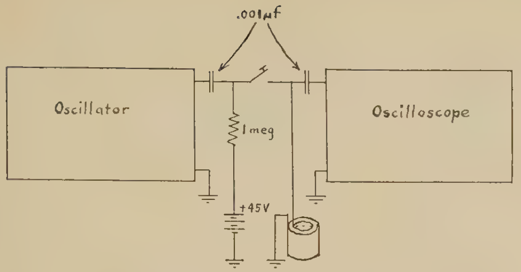

FIG. 1. Schematic of instrumentation employed in irradiation of specimens.

calibrated oscilloscope across the crystal enabled us to measure the signal amplitude and keep it constant at .25 volt at all frequencies. We explored the frequency range from .9 mc to 5.5 mc within which range the calculated sonic resonance of our paramoecia should lie. When using the crystal below its own resonance, the efficiency of transduction was probably very low. (As a check on the operation we drove the crystal at 10 mc and using a low power microscope we could see the paramoecia contort, distort, and die.) However, at frequencies within our experimental range, they appeared completely oblivious to the radiation. It was thus necessary to use physiological behavior as our observable and we selected reproduction as the simplest to observe.

As a check against possible mechanical resonances in the instrumentation itself, we measured the current to the crystal by means of the voltage drop across a series resistor. Using this value of current and the measured value of voltage across the crystal, we calculated the impedance of the crystal both at crystal resonance, and at all spot frequencies of the experiment. At crystal resonance, this impedance was 285 Ω. Over the entire frequency range of the experiment the impedance measured 670 Ω. Since there were no dips in impedance in the experimental range, we believe there existed no spurious mechanical resonances to obscure the response of the organisms. We also made an effort to measure the energy applied to the organisms by measuring the heat produced in the suspended drop using a delicate thermocouple. At crystal resonance we could detect a small fraction of 1°C temperature increase. Within the frequency range of the experiment, this was not detected.

As additional check against resonance in the instrumentation being mistaken for resonance in the test organism, we collected from a local lily pond, a strain of paramoecia whose mean length was 174 μ. Using the same instrumentation and the same method we found resonance response at approximately 4.7 mc/sec, a frequency which corresponds to the mean length, and we did not find resonance at the 4 mc/sec frequency as with *Paramoecium caudatum*. Due to difficulty in isolating and in culturing this local strain we dropped them as being unsuitable for our experiment. However, the partial results obtained with them serve to convince us that we are dealing with biological resonance.

We took our specimens from a culture in the logarithmic growth phase of its development. By daily inoculation of new cultures we were able to have one culture in the logarithmic growth phase at all times. We placed a drop of culture containing several specimens in the small cylindrical ceramic crystal where surface tension held it suspended. Every other drop was exposed for 5 minutes to the vibration, and the alternate drop was allowed to hang in the crystal for 5 minutes without exposure to be used as a control. At each frequency we exposed 10 drops and prepared 10 control drops. After exposure we carefully allowed exactly 5 paramoecia (under a microscope) to escape into a small amount of the culture medium in a ¾″ watch glass and placed these glasses in a moist, warm, illuminated location to incubate.

After 24 hours, we counted the number of specimens in each watch glass. For each frequency this gave us 10 exposed samples and 10 controls. We divided the mean of the number of specimens at the 24-hour level of the exposed samples by the mean of the corresponding controls, thus giving a ratio R of the multiplication rate of the exposed specimens to that of the controls. In the absence of effect due to exposure this ratio should be unity. We carried out this experiment for spot frequencies separated by .05 mc/sec over the frequency range from .9 mc/sec to 5.5 mc/sec and plotted the ratio R against frequency (Fig. 2). To give some idea of the variability

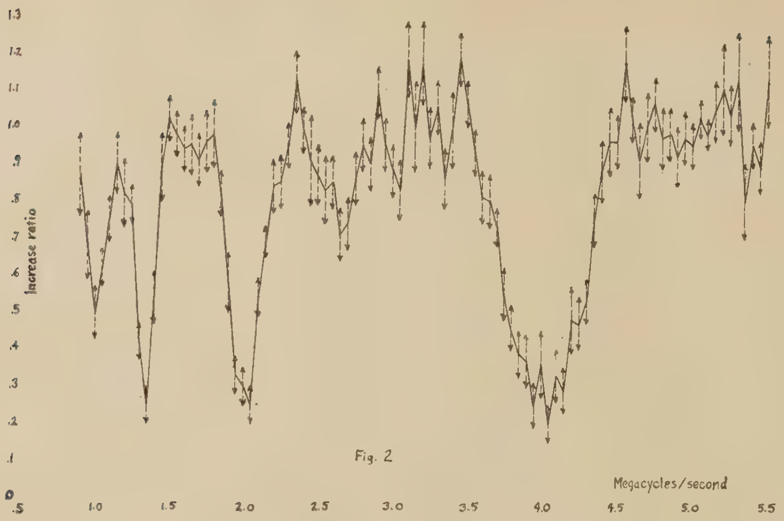

FIG. 2. Increase ratio vs frequency.

of the samples in any one frequency group we also calculated the standard deviation of the 10 exposed samples and indicated this value on the graph by vertical arrows each side of the value of R. The curve Fig. 2 indicates very clearly a resonance response at 4.0 mc/sec. An unexpected result was the response of the organism to the second harmonic content of a 2 mc/sec oscillation, the third harmonic content of a 1.3 mc/sec oscillation and even the fourth harmonic of a 1 mc/sec oscillation. This is still a response of the organism at 4 mc/sec. There may be present in the 2 mc/sec signal, for example, a certain percentage of 4 mc/sec harmonic, manifested as a variation from a true sine wave of the wave shape of the 2 mc/sec signal. Alternatively the 4 mc/sec response may be produced by the organism itself receiving a stimulus on alternate cycles. But wherever the 4 mc/sec harmonic is produced, either in the oscillator, in the transducer or in the organism, the apparent response of the organism to the subharmonic fundamental of 1 mc/sec, 1.3 mc/sec, 2 mc/sec, is in reality a response at 4 mc/sec and consequently a resonance response.

The frequency response of our oscilloscope amplifier did not allow us to work above 5.5 mc/sec. Therefore one of the questions awaiting answer by further experimentation is: Would the organism show resonance response by vibrating in segments, 2 segments at 8 mc/sec, 3 at 12 mc/sec, etc. Another question awaiting answer is: would there be a resonance response corresponding to other measurements of the organism besides its length, for example, its width.

The resonance method would appear to be useful for studying a number of interesting biological problems such as: (1) resonance of the individual cell within the body of a polycellular organism; (2) overall body resonance in a polycellular organism; (3) resonance of organs in higher animals, *e.g.* liver or brain; (4) development of a resonance spectrum for use in taxonomic determinations; (5) the study of the properties of protoplasm; (6) by the use of electrostriction to make organisms act as self transducers, extending the frequency range of supersonic vibration so that bacteria may be resonated, and possibly destroyed, retarded in development, encouraged in mutation, or even stimulated.

Summary. Samples of *Paramoecium caudatum,* length 207 ± 4.7 μ exposed to supersonic vibrations of low intensity at frequencies from .9 mc/sec. to 5.5 mc/sec. and allowed to multiply for 24 hours, show a response (reduction in multiplication rate) at the frequency expected by acoustic resonance principles, and also at subharmonic frequencies. Some suggestions for additional investigation are included.

A word of appreciation is due to the several technicians whose painstaking application collected these data, and to Dr. Charles Winter whose counsel helped materially.

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New Technic for Intracavitary Use of Co^{60}.* (21827)

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The tendency of bladder papillomata to recur following excision or fulguration and the difficulties involved in the management of carcinoma of the bladder by radical surgery impel one to exhaust all possible methods of treatment for this common neoplasm. Radiocobalt has been available from the Oak Ridge National Laboratory since 1937; however, relatively few of its medical potentialities have been explored until quite recently(1). Schulte *et al.*(2) attempted to use radiocobalt in the treatment of bladder neoplasms. They used a small "cobalt bead" ingeniously mounted in a specially devised Foley catheter for intraluminal irradiation. While their method appeared singularly unique, they nevertheless, after a trail on a small series of cases, declined to recommend its further use (2,3).

A new technic for the intracavitary administration of radiocobalt has been employed in a series of animals. A heavy metal precipitating agent and a wetting agent were used in conjunction with Co^{60} in an attempt to fix the radioactive substance at the tissue site and so prolong the radiation effects of the latter.

Methods and materials. Seven male and 6 female mongrel dogs were used in this experiment. The preoperative preparation consisted only in withholding food for 12 hours. Sodium pentobarbital anesthesia was used intravenously. After the animal was anesthetized, catheterization of the female was accomplished with a No. 14 Foley catheter; in the male a No. 6 or 7 French ureteral catheter was used. The bladder was evacuated and rinsed with sterile water. The first group comprised of 3 males and 3 females was used as a control group. Ten μc of Co^{60} in 10 ml of sterile water with 23 mg cobalt as carrier was instilled into the bladder. Five ml of sterile water was then injected into the catheter to clear it of radioactive material. The solution was allowed to remain in the bladder for one hour prior to withdrawal. Following this aspiration, the bladder was gently rinsed and the catheter removed.

The second group of 3 male and 2 female dogs was similarly prepared. After catheterization a solution containing 10 ml of 1% 8-oxyquinoline† and 10 ml of 1% d-sorbitol was instilled in the bladder of each animal and

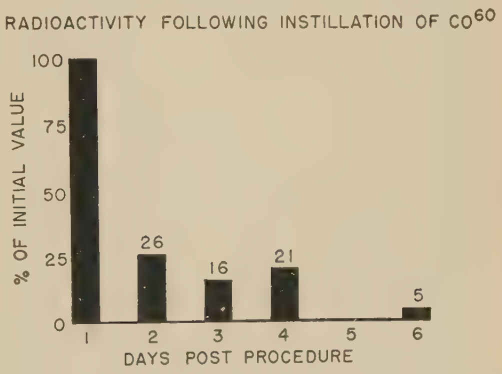

FIG. 1.

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* Supported in part by a grant from the Damon Runyon Memorial Cancer Fund (DRIR 230)

† 8-oxyquinoline forms an insoluble complex with Co^{++}.

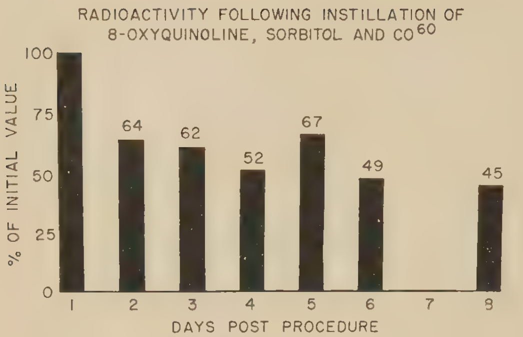

FIG. 2.

allowed to remain for 30 minutes. After evacuation of this solution, 10 μc of Co^{60} was introduced into the bladder lumen for one hour in an identical manner as described above. In 2 additional animals oxyquinoline alone was used before Co^{60}.

In the course of the 8 days following the procedure, radioactivity of the bladder was determined in a routine manner with a scintillation detector and conventional sealer. Three areas for radioactivity determination were selected in the midline of the suprapubic region 5 cm apart beginning at the symphysis and extending superiorly toward the xiphoid process.

Results. The first group of animals having received radiocobalt instillations into the bladder was found to have considerable radioactivity for one or 2 days. The average count per minute over background found 24 hours after instillation of radiocobalt was 4390. Forty-eight hours after the procedure the measured radioactivity had fallen to 26% of the initial 24-hour reading. On the third day the average recorded value was 16% of the first day's value. Further recordings showed progressive but slow diminution in the radioactivity. Six days after the procedure the average recording was only 5% of the initial value (Fig. 1).

The second group of 5 dogs exhibited a more prolonged radioactivity. Twenty-four hours after the procedure the mean activity was 3098 counts per minute over background. The 48-hour determinations averaged 64% of the initial count, and in the following 24-hour period the mean count remained a 62% of the first 24-hour value. Finally, 8 days after the procedure the average reading was 45% of the original determination (Fig. 2). The 2 dogs in which only oxyquinoline and radiocobalt were used showed some prolongation of the measured radioactivity.

Discussion. The application of radiotherapeutic agents on the surface of tissues has been limited by necrosis, infection and sloughing of the areas directly involved. The introduced concept utilizes a precipitating agent and a dispersing agent. Fixation of the radioactive isotope on the surface of the tissue should theoretically prolong the effectiveness of the radio-isotope. This was demonstrated under experimental conditions. The action of the dispersing agent further prolongs the effectiveness of the radiocobalt by increasing its penetration into the tissue. This diffusing action increases the area of distribution of the radio-isotope and should, theoretically, increase the efficiency while decreasing the untoward side-effects.

Summary. 1. A new concept is described for the intracavitary use of radiocobalt employing an agent to precipitate the cobalt directly on the mucosa of the urinary bladder together with dispersing agent to enhance penetration of the radiocobalt into the tissues. 2. Results in the use of this technic in dogs are presented. When 8-oxyquinoline solution was instilled into the bladder prior to the radiocobalt, there was an increase and prolongation of measureable radioactivity. When both 8-oxyquinoline and sorbitol were used this effect was further augmented.

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Effect of Feeding Fluoroacetate to the Rat.* (21828)

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Fluoroacetate has been shown to be an extremely toxic compound. As compound "1080" its use as a rodenticide was first reported by Kalmbach(1), and the LD_{50} dose for the white rat was found to be 2.5 mg of fluoroacetate/kg of body weight. They reported the development of a tolerance for fluoroacetate which was lost when feeding was stopped for 7 days. Buffa and Peters(2) first reported that the injection of fluoroacetate caused an accumulation of citric acid in various organs of the rat. Chenoweth(3) published a review on the toxicity, symptoms, and mechanisms for the toxic action of fluoroacetate.

It became of interest in relation to skeletal fluorine metabolism to determine the effect of sub-lethal dietary fluoroacetate upon bone fluorine concentration.

Methods. Twenty-one-day-old weanling female albino rats weighing 35 to 50 g were housed in colony cages on raised wire screen floors. They were allowed food and water *ad libitum*. Growth was followed by weighing each rat weekly. Sodium monofluoroacetate (Monsanto 90%) was ground in sucrose and then incorporated in the basal ration to give the desired concentration. The basal ration was composed of the following: 29 lb ground whole wheat, 25 lb ground yellow corn, 12 lb powdered skim milk, 10 lb soybean oil meal, 10 lb linseed oil meal, 8 lb alfalfa leaf meal, 5 lb melted butter, 1 lb calcium carbonate, and 1 lb iodized salt. The first experiment with 4 rats per lot was designed to determine the tolerance level of fluoroacetate. Exp. II was an attempt to test the tolerance concentration under varied conditions and to determine the adaptability of the rat to increased increments of fluoroacetate. The experimental setup for Exp. II is given in Table I. At the termination of the study the animals in Lots 1 (control) and 4 (20 ppm FA) of Exp. I and Lots 1 and 2 of Exp. II were sacrificed and their femurs removed for fluorine analysis. The bones were dehydrated and fat ex-

TABLE I. Effect of Feeding Fluoroacetate to the Rat.

		Weekly gain in g					
Period	Weeks	Lot 1 (5)†	Lot 2 (10)	Lot 3 (10)	Lot 4 (10)	Lot 5 (5)	Lot 6 (10)
		*0	20 ppm	0	20 ppm	0	20 ppm
I	1	18	5	18	8	16	2
	2	15	9	15	5	17	11
	3	23	12	23	8	22	13
	4	23	18	19	19	26	20
	5	13	14	16	11	14	13
	6	10	6	11	5	12	6
	7	5	11	10	11	2	8
		*0	20 ppm	40 ppm	40 ppm	0	0
II	8	20	12	–10	0	15	13
	9	14	8	8	13	11	21
		*0	20 ppm	40 ppm	40 ppm	20 ppm	20 ppm
III	10	5	8	8	10	–5	–7
	11	6	6	–1	5	6	9
	12	9	14	7	12	7	10

* Fluoroacetate added.
† No. in parentheses indicates No. of rats.

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TABLE II. Effect of Dietary Fluoroacetate upon Femur Fluorine of the Rat.

Lot	μg of F /femur	ppm of F ashed wt	Wks on exp.
1 Exp. I	10	73	9
4 I	15	146*	9
1 II	18	99	12
2 II	28	202*	12

* Highly significant difference Exp. I Lot 1, 4 and Exp. II Lot 1, 2.

tracted and then ashed at 550°C for 2½ hours. The fluorine content of the bones was then determined by the method of Willard and Winter(4) as modified by the Alcoa Research Laboratories(5).

Results. The inclusion of 5 to 10 ppm of fluoroacetate in the diet caused only a transient fluctuation in the growth rate of the rat. The addition of 20 ppm inhibited the growth rate sharply during the first week, an effect which gradually diminished in subsequent weeks. The results of Experiment II are summarized in Table I. It is evident that 20 ppm of fluoroacetate was again sufficient to sharply inhibit growth for several weeks. Nearly complete recovery of the growth rate was evident in 3 to 4 weeks. In period II the sudden introduction of 40 ppm of fluoroacetate into the diet not only sharply retarded growth but it caused a marked temporary loss in body weight. However rats accustomed to 20 ppm in their diet showed only a transitory fluctuation in the growth rate during the first week after their dietary fluoroacetate was increased from 20 to 40 ppm. The reintroduction of 20 ppm of fluoroacetate into the ration after a 2-week period when the toxicant was not fed (Lot 5 and 6) elicited the same kind of response as that observed in the rat first exposed to the compound.

The femuri of the rat increased in their fluorine content when 20 ppm of fluoroacetate was incorporated in the diet for 9 to 12 weeks (Table II). The basal diet contained approximately 5 ppm of fluorine. The fluorine content of fluoroacetate is 24% hence the inclusion of 20 ppm (fluoroacetate) supplied approximately 5 ppm of fluorine in addition to that present in the basal ration which was fed to the control animals in Lot I of both experiments. These data show that the dietary F and the supplemental fluoroacetate F were equally available for deposition in the skeleton of the rat. Since the added fluoroacetate F increased the F ingestion by a factor of 2 it would be expected that femur storage would therefore be doubled. This was demonstrated to be the case. Further these data add more evidence to show that small amounts of ingested F caused a distinct and measurable increase in bone F. The deposition of F in the bone is thus a function of dietary intake and also a function of the length of the period of time of exposure to it. The effect of the time element was demonstrated in these studies since there was an increased femur F (on an ash wt. basis) between control animals removed from experiment at the end of 9 weeks and those removed at the end of 12 weeks. In the case of the former the femuri contained on the average 77 ppm F while those from rats maintained for 12 weeks averaged 99 ppm F. A similar difference was shown by the rats fed fluoroacetate for 9 and 12 weeks in which they stored in the femuri 146 and 202 ppm F respectively.

Summary. The tolerance to dietary fluoroacetate and its effect upon skeletal fluorine deposition in the rat has been studied. It has been shown that the rat will survive the addition of 20 ppm of fluoroacetate to the diet, that there was a period of adjustment to it during which time there was a sharp retardation in growth rate, that sufficient physiologic adjustment was made to recover normalcy, that exposure to the toxicant conditions the animals to further increases of dietary fluoroacetate with only transitory outward effects and that this conditioned physiologic tolerance was quickly lost. Further it has been shown that there was an increased storage of fluorine in the femuri when dietary fluoroacetate was fed. It appears that fluoroacetate contributed an amount of fluorine to the femuri equivalent to that contributed by the basal diet.

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A Nylon Cloth Oxygenator for Extracorporeal Circulation.* (21829)

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Film oxygenators are preferred to bubble oxygenators because of the difficulties incident to the removal of minute bubbles from the blood(1-8). In the search for a film oxygenator as efficient and compact as possible, the use of a steel screen(9), or cloth mesh (10) seems advisable since both surfaces of the film can be exposed. Cloth, furthermore, can be easily washed and is now available in biologically inert forms such as nylon(11). A fine material results in a thin film with minimal holdup. Turbulence of the film is achieved by the cross threads.

Investigation of the critical range of size of the mesh openings reveals that with mesh openings too large the film becomes discontinuous, with mesh openings too small the film is thicker. After testing several materials sheer nylon stocking (50 denier) and pretreated(11) nylon tulle (15 denier) were found to have the best filming qualities. The openings in the latter are approximately 2 mm in diameter and hexagonal. The stocking is difficult to use because of its elastic properties.

Apparatus. The first oxygenator (Fig. 1) consists of a plastic frame supporting a nylon curtain in the shape of a cylinder, 7 inches (17.8 cm) in diameter, and 12 inches (30.5 cm) high. The cylinder is rotated within a plastic container by a small electric motor at 60 revolutions per minute. Warmed, humidified oxygen at 40 Lit. per min. without carbon dioxide is supplied to the inner and outer surfaces of the film. Considering both sides the oxygenating surface is approximately 2 square feet (1.07 m^2).

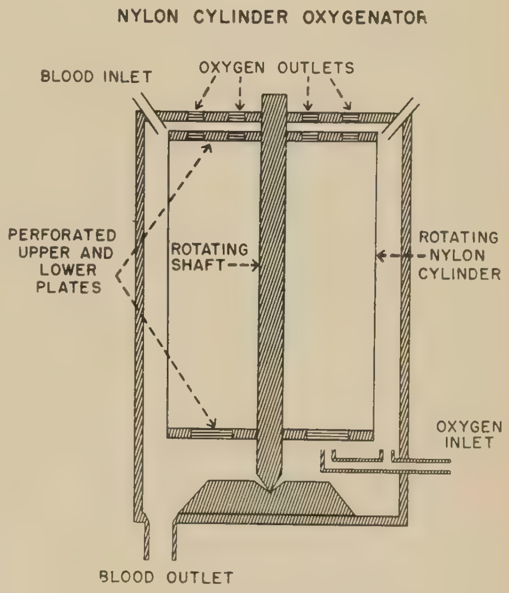

FIG. 1.

Results. Applying the principle of work function units as described by Gimbel and Engeberg(4) to the data presented in Table I the oxygenating capacity of this device is equivalent to raising 964 cc of blood per minute with a hemoglobin of 15 g from a venous saturation of 60% to an arterial saturation of 90%. The oxygen uptake at 1 liter of blood flow per minute is 43 cc per minute. A higher capacity oxygenator with multiple concentric cloth cylinders is now being tested.

Analysis of the carbon dioxide tensions, which was routinely done, showed a drop in

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* This work was supported by the Mary Bailey Foundation for Heart and Great Vessel Research, Philadelphia, Pa.

TABLE I. Oxygenating Capacity of Nylon Cloth Cylinder.

Blood flow, cc/min.	Blood O_2 capacity, vol %	Oxygenation: Venous sat., %	Oxygenation: Arterial sat., %	A. V. diff., %	O_2 absorbed, cc/100 cc blood	O_2 absorbed, cc/min.
900	10.1	54.7	104.0	49.3	4.97	44.8
800	10.8	53.1	97.0	43.9	4.74	37.9
1000	12.5	65.3	99.5	34.2	3.89	38.9
950	10.1	60.3	95.4	35.1	3.57	33.9
Avg 913	10.9	58.4	99.0	40.7	4.29	38.9

the average venous tension to 25.0 volumes % and in the arterial to 20.3 volumes %. With an efficient oxygenator of this type it appears that it is necessary to add carbon dioxide to the infused oxygen(2).

The amount of blood necessary to form a complete film over the entire cloth is 40 cc. At a flow rate of one liter per minute, the amount of blood on the curtain is 100 cc. The tubing and collecting pool require an additional 200 cc making a total holdup(4) of 300 cc. The film thickness as determined by the amount of blood on the cloth divided by the area is approximately 1 mm. The time the blood takes to traverse the nylon cloth is approximately 5 seconds. The comparative efficiency (oxygenating capacity divided by holdup) is 3.

To vastly increase the surface area, a second model filming in 3 dimensions was designed and tested (Fig. 2). Filming in this model occurs between the layers as well as on both surfaces of the many layers of nylon tulle rolled loosely around a central perforated shaft through which oxygen is passed. The roll of nylon was 14 inches (35 cm) high, and 5 inches (12.5 cm) in diameter with a 1.5 inch (3.8 cm) central shaft. The blood is evenly distributed by rotating inlets at the top and passes directly from the base to a small reservoir. No bubbling or frothing occurs with an oxygen flow rate of 15 liters per minute.

ROLLED NYLON OXYGENATOR

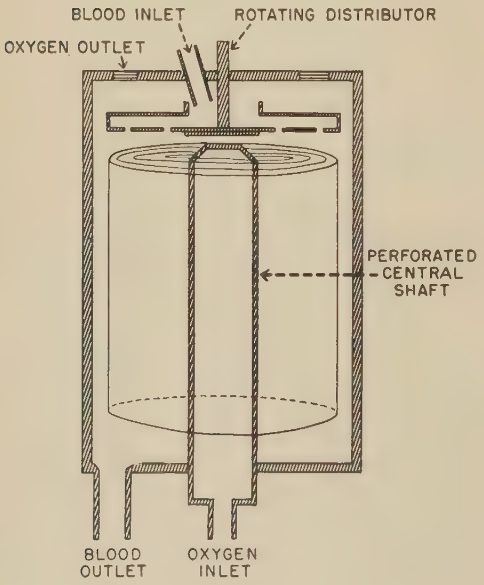

FIG. 2.

Oxygen uptake with this second device is shown in Table II. The oxygenating capacity calculated using work function units is 1230 cc per minute under standard conditions(4). The average venous carbon dioxide content was 39.4 volumes %, and the average arterial 27.2 volumes %. At a flow rate of one liter of blood per minute, the amount of blood in this mesh is 300 cc. An additional 200 cc in the collecting reservoir brings the total holdup to 500 cc. The comparative efficiency is 2.5. Although routine hematologic studies were not performed a sample determination before and after 30 minutes of perfusion at one liter per minute showed no significant change in the number of red or white cells or in the platelet count. Little hemolysis was evident on centrifugal blood specimens.

Discussion. The results show that both of the nylon mesh oxygenators are efficient and have capacities adequate for experimental animal use. In addition they are compact, easy to clean, have minimal holdup, little hemolysis, and function without foam or fibrin formation(12,13). Consequently little heparin is required during their use. We have used 2 mg per kilogram of the dog's weight for both

TABLE II. Oxygenating Capacity of Rolled Nylon Cloth Oxygenator.

Blood flow, cc/min.	Blood O_2 capacity, vol %	Oxygenation		A. V. diff., %	O_2 absorbed, cc/100 cc blood	O_2 absorbed, cc/min.
		Venous sat., %	Arterial sat., %			
800	18.9	29.2	74.9	45.7	8.64	69.2
1200	19.5	49.5	100.6	51.1	9.97	119.5
1250	19.7	45.5	79.7	34.2	6.75	84.4
650	18.2	37.9	96.7	58.8	10.70	69.6
Avg 950	19.1	40.0	88.0	47.5	9.02	85.7

donors and perfused animals without clotting.

The last 10 consecutive dogs in whom the nylon roll with a Gemeinhardt pump(14,15) was used for complete heart and lung bypass survived the perfusion. All of these were subjected to thoracotomy with vena caval occlusion around cannulae and azygos ligation for 15 to 30 minutes. The last 6 in addition had their cuffed, inflated endotracheal tubes clamped. A blood rewarming device was routinely used to maintain normal temperature. Seven dogs lived for 5 days or more without evidence of cerebral or visceral damage. Three died of causes incident to the thoracotomy at 2, 4 and 5 hours, postoperatively. Of the 4 dogs that were perfused for 30 minutes, 3 have survived to date.

Summary. Two experimental oxygenators employing nylon cloth for filming and oxygenating blood have been described. Preliminary tests show that this type of oxygenator has a high comparative efficiency.

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Thyroid Uptake of Radioactive Iodine in the Dog; Effect of Diet, Hypophysectomy, and TSH.*† (21830)

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(Introduced by George W. Thorn.)
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Because it is difficult to produce myxedema in the dog by surgical thyroidectomy, and because plasma protein-bound iodine levels are low in this species(1), it has been claimed that the dog is uniquely less dependent on its thyroid for normal metabolic activities than other species(2,3). Goldberg and Chaikoff (4) were able to show, however, that radiothyroidectomy would produce typical myxedema in the dog when dietary thyroxine intake was curtailed. It has been found in this laboratory that when the dietary iodine intake is restricted, the normal dog has an appreciable thyroidal uptake of iodine, and that this uptake is depressed by hypophysectomy and stimulated by thyrotropin administration. These studies were performed in the unanesthetized dog, using a specially constructed scintillation counter.

Methods. The scintillation counter¶ (Fig. 1) employed a thallium-excited sodium iodide crystal, 1½ in. in diameter and 1 in. thick, an RCA 5819 photomultiplier tube, and a lead shield of 1 in. minimum thickness, with a tapered bore. The top of the shield was fitted with a groove fixing the neck 7 in. above the crystal face. At the bottom of the groove, 1 μc of I^{131} counted approximately 5000 c.p.m. The top of the mounted shield was 20 in. from the floor, an average neck-to-floor height for most dogs used. Blood samples were counted in 20 ml beakers placed directly over the crystal and appropriate correction was applied to relate the radioactivity obtained to that predicted for the neck level. Five to 10 μc of carrier-free I^{131} in saline (obtained from Oak Ridge, Tenn. through the Harvard Biophysics Laboratory) were injected intravenously. No correction for residual radioactivity in the syringe was found to be necessary. The thyroid was monitored by extending the neck of the dog over the shield of the counter, taking care to avoid tracheal compression. Maximum counts were obtained when the anterior edges of the transverse processes of the second cervical vertebra were held tangent to the edge of the shield. A glass thyroid phantom containing I^{131} was counted first in the air at the neck position, and then after implantation in the thyroid area in a series of cadavers. The counting rate did not differ in the two locations, and hence, no correction for back scatter was employed. An area of the chest, 4 cm above the tip of the xiphoid, which avoided the stomach, was counted as an indifferent area. To relate counts in this area to non-thyroidal neck counts, neck and chest counts were followed for 5 days after administration of I^{131} in 8 surgically thyroidectomized dogs. The mean ratio of neck c.p.m. to chest c.p.m. was established as 0.7 at 24 hrs after injection of the tracer, 1.0 at 48 hrs, and 1.1 thereafter. In addition, 4 normal dogs were given I^{131}, and 1-8 days later the neck and chest were counted before and after removal of the thyroid. These values confirmed the ratios cited above. There was no late rise in neck counts in the dogs initially thyroidectomized to suggest incomplete removal of the gland. The

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† These studies were conducted under Atomic Energy Commission Authorizations.

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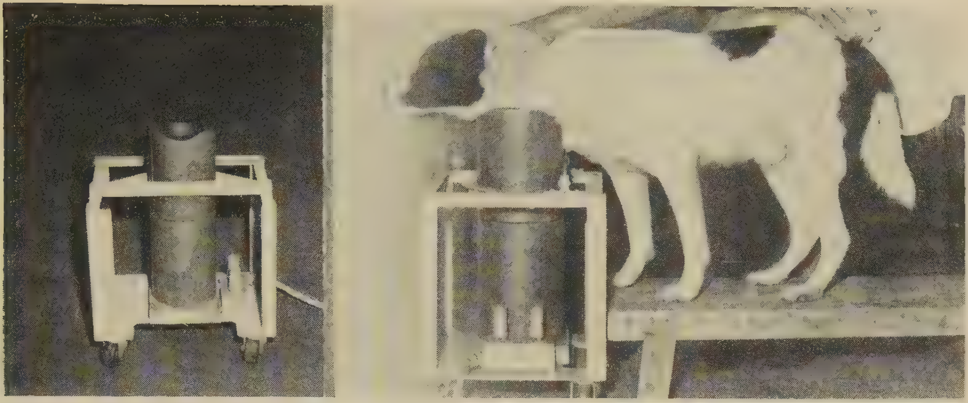
¶ The counter represents an adaptation of an original design by Dr. Gordon Brownell and Mr. Robert Cavicchi of the Physics Laboratory, Massachusetts General Hospital. The authors are indebted to Dr. Brownell for many helpful suggestions and to Mr. Cavicchi for much of the instrument construction.



A B

FIG. 1. A—Scintillation counter for dog use. Photomultiplier tube is fitted in the base of the lead cylinder, with the crystal resting on top of it. Preamplifier is at right of shield. B—Counter in use. Dog is held so that the anterior edges of the transverse processes of the axis are even with the edge of the lead shield.

changing ratios were due rather to a faster decline in chest counts. Neck counts were therefore corrected for non-thyroidal activity by subtracting the product of the chest counts and the ratio appropriate for the time elapsed after I^{131} injection. The mean variation between repeated neck counts was 1.5% of the total count, providing the counting rate was greater than 400 c.p.m. The mean variation in repeated chest counts was 2.4%. Uptakes were further corrected for decay and instrument variation. Adult mongrel dogs of both sexes weighing 9 to 15 kg were utilized. They received a ration (Ken-L-Biskit)** which provided 50-70 μg of iodine/day. Thyrotrophic hormone (lot No. A-k41006R) was kindly supplied by the Armour Laboratories. Hypophysectomies were evaluated by microscopic examination of the serially-sectioned sellar area, and all were found to be complete, except for small remnants of the pars tuberalis in one dog.

Results. In Fig. 2, the mean values for the % of the tracer dose present in the thyroid gland 24, 48 and 72 hours after injection are presented. In 26 apparently normal animals, the uptake rose for 3 days, then levelled off before beginning a slow exponential decline on the 5th to 7th day.

The effect of dietary iodine on the thyroid uptake of the dog was demonstrated in 3 dogs by obtaining uptake values before and after 1 to 2 weeks on a ration containing 0.003% potassium iodide. In all animals, uptake was depressed from normal values to less than 7% at 72 hrs (Table I).

In Fig. 3A, data is presented on 4 animals counted daily for 3 weeks Straight lines were fitted to the decay curves by the method of least squares and half-life values computed from the slopes. The half-life values obtained in a series of 8 normal dogs by this method ranged from 20.4 to 36.6 days (mean, 28.8 days). These figures do not compensate for

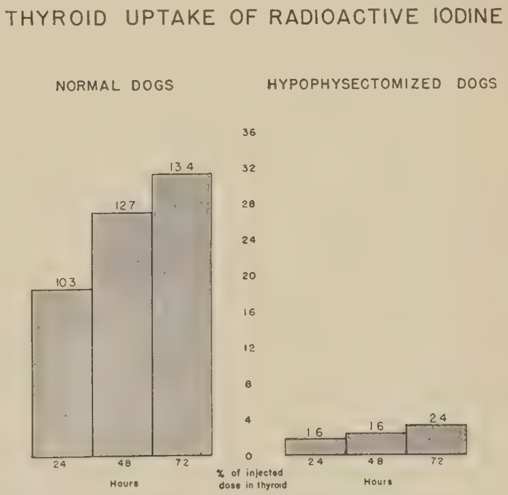


FIG. 2. Uptake values are means. Figures at top of each column are stand. deviations.

** Quaker Oats Co., Chicago, Ill.

TABLE I. Effect of High Iodine Diet on % Uptake of I^{131} by Thyroid in the Dog.

Dog	220		221		228	
Dietary iodine	Low	High	Low	High	Low	High
Day 1	30.3	1.6	13.1	.7	23.2	3.9
2	45.0	3.3	17.9	1.8	35.9	5.0
3	48.3	4.4	22.1	5.9	40.4	6.2

reutilization, since no blocking agents were used.

In Fig. 3B, the effect of TSH administration on the shape of the decay curve is illustrated. The more rapid release of I^{131} containing material from the gland is consistent and reproducible. When TSH was administered before tracer injection, the initial uptakes were also higher than normal and reached a maximum earlier. When uptakes with and without TSH administration were compared in 4 dogs, the increases in maximum uptake were 47, 100, 111, and 155%, and the maximum uptake was reached on the first day in three dogs, and on the second in the fourth. On the other hand, when repeat uptakes were performed in 8 normal dogs, the variation between uptakes averaged 23% (Table II).

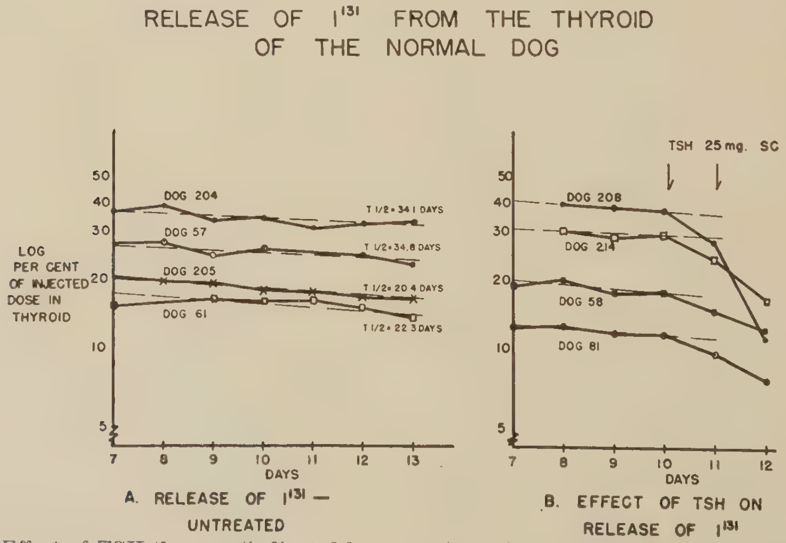
The mean uptake of I^{131} determined in 6 dogs 33 to 200 days after hypophysectomy is shown in Fig. 2. The depression for the group at each time interval is highly significant, when compared to the normal.

Blood I^{131} levels were determined at intervals during the first 30 hours after tracer injection. Thyroid I^{131} clearances were calculated in the conventional manner(5). The rates were consistent over the first 24 hours in the individual dogs. The slow rate of conversion of plasma inorganic iodide to the organically bound form permits use of whole blood rather than protein-free extracts for clearance measurements during this period. A comparison between values in normal and hypophysectomized animals is presented in Table III.

Discussion. A short-term study of the kinetics of I^{131} metabolism has been reported for the anesthetized dog(6), but a reproducible, simple technic of *in vivo* counting has apparently not been previously reported.

It is of interest that the maximum thyroid uptake is attained slowly in the dog, not before the third day. This is quite in contrast to the rabbit in which percentage uptake is greatest at 24 hours(7). However, the average % of the tracer eventually detected in the dog thyroid is essentially the same as that in the rabbit and the human.

That the level of thyroid activity in the dog



A. RELEASE OF I^{131} — UNTREATED

B. EFFECT OF TSH ON RELEASE OF I^{131}

FIG. 3. Effect of TSH therapy (indicated by arrows) on the release of radioactive iodine from the thyroid. Biologic half life (T½) values are calculated from the fitted straight line decay curves.

TABLE II. Reproducibility of Repeated I^{131} Uptakes in 8 Normal Dogs.

Days between 1st and 2nd uptake	% uptake at 72 hr 1st	2nd	% variation*
24	24.9	27.8	11.0
26	34.5	24.2	35.0
"	18.2	28.2	43.1
24	38.6	31.7	19.6
8	40.1	46.6	15.0
16	14.5	14.7	1.4
"	20.2	25.6	23.6
"	19.8	28.0	34.3
Mean	26.3	28.3	22.9 ± 13.9†

* $\frac{2-1}{\text{mean of } 1+2} \times 100$, ignoring signs.

† Stand. dev.

is under the control of the pituitary is clearly indicated by the 9-fold drop in uptake that occurs after hypophysectomy, and the marked increase in uptake and turnover rate that follows TSH administration.

Monitoring the gland *in vivo* by the present technic is easier than obtaining accurate urine collections for calculation of the thyroid uptake, although such data, especially when accompanied by stable iodine analyses, would add to the completeness of testing(8). However, for the purposes of screening a series of dogs, it appears that *in vivo* monitoring alone is sufficient to detect major changes in thyroid function.

TABLE III. Comparison of Clearances and Uptakes in 3 Normal and 2 Hypophysectomized Dogs.

	Clearance, cc whole blood/min.	% uptake at 48 hr
Normal	1.63	29.7
	.81	18.4
	1.76	29.5
Hypophysectomized	.16	3.9
	.09	3.1

Summary. A scintillation counter adapted to the determination of thyroid I^{131} uptakes in the unanesthetized dog has been described. The dog was shown to have a relatively slow rate of accumulation of I^{131} by the thyroid, but a total uptake of the same order of magnitude as man and the rabbit if the dog was maintained on a dietary intake of 70 μg of iodine or less per day. Hypophysectomy markedly depressed the thyroidal uptake and blood I^{131} clearance rate, and TSH administration elevated the rate of uptake and release.

We would like to thank Miss Hildegard Junker, Miss Faith Bevchick and Miss Ethel Hitchcock for technical assistance. Dr. George W. Thorn kindly furnished some of the radioactive iodine and the TSH used in these studies.

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Effect of Dietary Choline, Methionine, and Vit. B_{12} on Weight and Composition of Eggs.* (21831)

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The correlation between the composition of the diet and that of the egg has been studied by several workers. Data have been presented (1) indicating that the egg protein of pigeons might be modified by the diet fed the birds and the cystine and methionine content of hen's eggs have been modified by dietary means(2). In contrast to these reports, McFarlane *et al.*(3) reported no change in egg proteins as a result of dietary modification. Titus *et al.*(4) were unable to affect the fat content of eggs by dietary means and Ingram *et al.*(5) were unable to change the tryptophan, lysine, cystine, or methionine composition of eggs by varying the composition of the rations. Savage *et al.*(6) found no difference in egg weight due to vit. B_{12}. The studies reported herein are the results of choline and fat analyses of eggs from hens that received a choline-deficient basal diet with or without additional methionine, choline or vit. B_{12} supplements.

Methods. New Hampshire Red chicks were fed the experimental diets for a period of 50-60 weeks before eggs were collected for analyses. (This experiment was designed primarily for tumor production studies in chronic choline-deficient chickens.) The basal diet consisted of the following ingredients (%): methanol-extracted soybean oil meal 40, salts 5.5, lard 19, cod liver oil 1, L-cystine 0.1 and sucrose to 100. The following amounts of vitamins were added (mg/kg of diet): pyridoxine 4, 2-methyl-1, 4-naphthoquinone 5, riboflavin 6, thiamin 6, Ca pantothenate 20, niacin 50, inositol 1,000, folacin 2, biotin 0.25, α-tocopherol 50, and α-tocopherol acetate 50. This diet was varied with respect to methionine, choline, and vit. B_{12}. The diet of control birds was supplemented with 0.3% choline chloride throughout the experiment. The choline in the diet fed the other birds was progressively decreased at 4-week intervals from 0.2% to 0.1% to .05% and no choline was added after the 12th week. Eggs were boiled and their yolks analyzed for choline by the method of Ackerman(7). The method is briefly described as follows: a 0.5 g sample was hydrolyzed with 15 ml of 25% HNO_3 for 5 hours. After cooling, the hydrolysate was filtered into a 50 ml volumetric flask and made to volume with water. A 20.0 ml aliquot was transferred to a 50 ml flask and the yellow solution neutralized with NaOH until alkaline to phenol red. Then 0.4 ml of 10 N NaOH was added. The solution was chilled at 5°C and 2.5 ml of a 5% ammonium reineckate solution in methanol was added and allowed to stand for 2.5-3 hours at 5°C. The reineckate crystals were filtered onto a pad of acetone-washed super-cel in a sintered glass funnel of medium porosity. Eight ml of n-propanol in 2 ml portions was used to wash the suction flask and walls of the funnel. The excess propanol was removed by aspiration. The reineckate crystals were dissolved in acetone and made to volume in a 5 or 10 ml volumetric flask. The optical density of the acetone solution was measured at 526 mu in a Beckman spectrophotometer. A molar extinction coefficient of 111 for choline reineckate was used to calculate the amount of choline chloride present(8). Recovery of choline added to experimental samples ranged from 97.8 to 106.4% with an average of 100.8%. Samples of the egg yolks were crumbled and dried 24-36 hours at 100°C. They were then extracted with ether for 12 hours in a Nolan fat extractor to determine the total amounts

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TABLE I. Effect of Choline, Methionine and Vitamin B_{12} on Egg Size and Composition.

				Yolk analysis			
Additions/kg basal diet	No. eggs analyzed	Egg wt avg, g	Yolk wt avg, g	Choline, mg/g	Total choline, mg	Water, %	Ether extract, % dry basis
0	11	51.9	12.6	10.5	132	50.8	49.3
1 g methionine	10	52.9	15.5	12.0	186	52.8	57.2
5 g "	6	48.9	15.7	12.9	203	51.4	59.7
10 g "	10	49.8	17.7	12.7	225	49.0	60.3
30 γ vit. B_{12}	10	63.0	20.0	13.3	266	48.3	63.0
3 g choline chloride	10	60.8	20.0	13.0	260	49.1	65.1
Commercial eggs	10	57.0	19.0	13.0	247	49.0	62.0
Least significant diff. ($p = .05$)		3.4	1.8	0.5	33	—	1.0
Coefficient of variation		11.3%	12.8%	9.5%	25.5%	—	9.6%

of ether extractable lipids present.

Results. The data concerning egg weights and composition are shown in Table I. Supplements of methionine to the choline-deficient diet increased yolk size (5.1 g or 40%), choline concentration (2.2 mg/g or 21%) and total choline (93 mg or 70%). Methionine had no effect on egg size. Supplements of choline increased egg size (8.9 g or 17%), yolk size (7.4 g or 59%), choline concentration (2.5 mg/g or 24%), and total choline (128 mg or 97%).

Supplements of vit. B_{12} increased egg size (11.1 g or 21%), yolk size (7.4 g or 59%), choline concentration (2.8 mg/g or 27%), and total choline (134 mg or 102%). There was a gradual increase in the fat concentration of the yolk as the amount of methionine was increased in the diet and a still greater increase resulted when choline or vit. B_{12} was fed. Analyses on some commercial eggs are shown for comparison.

Discussion. The interrelationship of vit. B_{12}, choline, and methionine in the chick has been demonstrated by several workers(9-11). Schaefer *et al.*(9) showed that the choline requirement for normal growth of chicks fed the basal diet without vit. B_{12} was 0.6% choline chloride as compared to 0.1% when vit. B_{12} was added to the diet. Savage *et al.*(6) observed no increase in egg weight when the rations were supplemented with vit. B_{12}. However, the rations contained adequate choline and the birds were fed a ration that contained fish meal for several weeks; a considerable amount of vit. B_{12} was probably stored during that period.

The data presented herein further demonstrate the interrelationship of choline, vit. B_{12}, and methionine in the laying hen. Although the choline concentration in the yolk was increased by only 24-26% when the diets were supplemented with choline or vit. B_{12}, the increase in the size of the yolks was sufficient to double the total choline in the yolk.

Summary. Choline deficiency in the laying hen resulted in smaller eggs and reduced concentration of fat (ether extract) and choline in the egg. Supplementations of choline or vit. B_{12} markedly increased egg size, choline and fat concentrations. Methionine increased yolk size, choline and fat concentrations but had no effect on egg size.

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Capacity of the Rat Intestine to Absorb Cholesterol. (21832)

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It has been shown that the chemical nature of the dietary lipid has a definite effect on the extent of the intestinal absorption of cholesterol(1,2). Among the derivatives of fat added to the diet, oleic acid was most active in increasing cholesterol absorption. For this reason oleic acid was used as the lipid in this study of the capacity of the rat's intestine to absorb cholesterol. According to our search of the literature, no study has been made in any species to determine the capacity of the intestine to absorb cholesterol.

Method. Twenty-four male rats of the Holtzman-Rolfsmeyer strain weighing about 250 g were used in this study. Each rat was caged individually. A basal diet of the following composition was given: Cerelose, 266.0 g; Casein (Nutritional Biochemicals Co.), 115.0 g; Salt mixture (Wesson modification), 25.0 g; Yeast (Pabst Co.), 37.0 g; Celluflour, 31.2 g, making a total of 474.8 g. Every 2 days 19.2 g of the above mixture was given to each rat, at which time 2 g (2.2 cc) of oleic acid and one drop of cod liver oil was added. One group of 6 rats received only the basal diet plus oleic acid. To the 3 remaining groups of 6 rats each cholesterol was added at 3 dose levels 96, 192 and 288 mg daily. Fecal cholesterol was extracted as in previous studies(1,2) and determined by the method of Kingsley and Schaffert(3). The animals were fed the 3 dose levels of cholesterol with identical basal diets for 40 days. During the last 4 days of the feeding period the feces of 2 rats were combined and analyzed for total lipid, unsaponifiable material and cholesterol.

Results. The values for fecal analyses are presented in Table I. In the same table, the unpublished data of Kim and Ivy(1) are also included. The latter study was not primarily

TABLE I. Results of Study of Intestinal Absorption and Fecal Excretion of Cholesterol when Fed Increasing Amounts of Cholesterol.

	Results of present authors (1954),‡ mg				Unpublished results of Kim & Ivy (1952),‡ mg		
	B.D. 9.6 g				B.D. 9 g	B.D. 10 g	B.D. 8 g
Amt cholesterol fed each rat daily	Control 0	96	192	288	Control 0	50	100
Total fecal lipid elimination	* 875 ± 53 † (109)	1312 ± 73 (164)	2153 ± 11 (269)	2949 ± 14 (369)	764 ± 73 (95)	1026 ±310 (124)	1321 ±151 (165)
Unsaponifiable material	* 608 ± 34 † (76)	662 ± 14 (83)	1540 ± 69 (193)	2395 ± 54 (299)	—	—	—
Total steroids: (Digitonin)	* 233 ± 34 † (29)	531 ± 12 (66)	1428 ± 14 (178)	1937 ± 53 (242)	151 ± 21 (19)	320 ± 44 (40)	541 ± 78 (68)
Cholesterol	* 121 ± 7 † (15)	422 ± 6 (53)	928 ± 56 (116)	1680 ± 15 (210)	69 ± 12 (9)	210 ± 38 (26)	426 ± 59 (53)
Cholesterol eliminated when corrected for control endogenous excretion	* 0 †	301 (37)	807 (101)	1559 (196)	0	141 ± 38 (17)	357 ± 57 (45)
% & mg cholesterol absorbed	—	61% 59 mg	47% 91 mg	32% 92 mg	—	65% 33 mg	55% 55 mg

* For 8 rat days. † For 1 rat day. ‡ Each dietary group received 1 g oleic acid.
B.D. = basal diet.

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[*] China Medical Board Fellow, Rockefeller Foundation.

[†] Rockefeller Foundation Fellow.

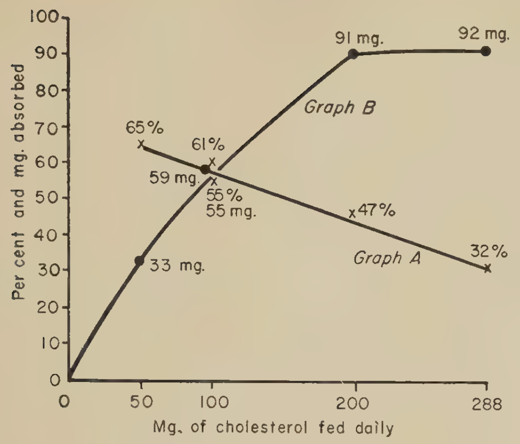

FIG. 1. Milligram and percentage of cholesterol absorbed in relation to milligrams of cholesterol fed daily.

designed to test the capacity of cholesterol absorption. However, it was performed under very similar dietary conditions but at a lower dietary cholesterol range, and the values quoted from Kim and Ivy were not values from the same series of experiments. It is interesting to note the close agreement of the results, as is evinced by the graphs in Fig. 1.

In Graph A. Fig. 1, the percentage of absorption decreases directly as the amount of dietary cholesterol is increased. This is also indicated by Curve B where a plateau is reached when the dietary cholesterol level reaches 192 mg per rat per day. At the 192 mg and 288 mg per rat day dietary cholesterol levels, the amounts of cholesterol absorbed were almost identical, namely, 91 and 92 mg, respectively. This leveling off of cholesterol absorption certainly indicates that the intestinal capacity for cholesterol absorption has been reached.

The existence of such a capacity is not surprising from a physiological point of view. That it exists has to be considered in the design and the interpretation of the results of experiments on the intestinal absorption of cholesterol and related sterols. It should be determined in those species in which cholesterol metabolism is under study. One of the rather obvious points of significance of the existence of a capacity for the mechanism for cholesterol absorption is that when a sterol mixture is fed the different sterols may compete for absorption on the basis of their relative absorbabilities.

Summary. The existence of a limited capacity for cholesterol absorption in the rat has been demonstrated. At dietary cholesterol levels of from 50 to 288 mg per 250 g rat per day the percentage of cholesterol absorption decreased as a linear function. The amount of cholesterol absorbed reached a plateau when the dietary cholesterol intake was 192 mg per 250 g rat per day. Under the conditions specified, the capacity of the intestinal mechanism for the absorption of cholesterol in a 250 g rat is 90 mg or approximately 360 mg per kilo body weight per day.

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Experimental Nephrotic Syndrome Induced in Rats by Aminonucleoside. Renal Lesions and Body Electrolyte Composition.* (21833)

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(Introduced by S. Farber.)

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Daily subcutaneous injection of 6-dimethylamino purine, 3-amino-d-ribose, an aminonucleoside related to "puromycin,"‡(1) into immature male rats for 10 to 12 days results in generalized edema, ascites, marked proteinuria, hypoproteinemia, hyperlipemia, and azotemia. The clinical complex and renal pathology closely resemble the nephrotic syndrome occurring in children. This initial report describes the renal lesions and electrolyte composition of edematous tissues in the rat. The observations indicate that the renal lesion involves both the glomerulus and the convoluted tubule; that skin, an essentially extracellular phase tissue, is the largest tissue reservoir for edema fluid; and that the electrolyte structure of muscle cells is only slightly altered during edema.

Procedure. Thirty-six 40 g male rats of Sprague-Dawley strain were caged individually and pair-fed on a standard synthetic diet containing 16% W/W vitamin-free casein. After a preliminary adjustment period, 18 rats (experimental group) were injected daily with 0.003 ml of a 0.5% aqueous solution of the drug per gram of body weight for 12 days. The control rats were injected with similarly calculated doses of distilled water. Twelve animals in each group were given an oral load of M/1 NaCl solution daily by stomach tube in a dose of 1 mM/100 g of body weight for the last 4 days of injection. All animals were sacrificed 24 hours after the last injection. At the time of sacrifice under Evipal anesthesia the hair was carefully shaved from the animals. Ascitic fluid, present in all experimental animals, was aspirated as completely as possible. All animals were killed by exsanguination from the abdominal aorta. Samples of lumbar and thigh muscle and the entire skin, except for paranasal and paratarsal attachments, were removed for analysis. In addition, the residual carcasses of 3 animals in each group, 12 in all, were analyzed. For determination of water and fat, tissues were dried to constant weight at 105°C and fat was extracted 4 to 8 times, about 12 to 18 hours per extraction, with a mixture of petroleum and ethyl ethers. A 0.75 N•HNO_3 extract of the defatted pulverized tissue was analyzed for sodium and potassium by flame photometry(2), and chloride was determined iodometrically(3). Total nitrogen content of the dried defatted tissue was determined following micro-Kjeldahl digestion by a modified microdiffusion procedure(4). Collagen nitrogen was also determined by microdiffusion analysis of a water extract of tissue, prepared as described by Eichelberger, Eisele and Wertzler(5).

Results. Mean body weight at sacrifice of the 6 edematous rats was 132 g (109-134.3). That of the 12 NaCl-loaded edematous rats was 153.15 g (113.8-189.1). Weights of similar groups of pair-fed control rats were 101.9 (90.2-114.8) and 110.8 (88-128.5).

In the experimental animals the renal glomeruli had a decreased number of erythrocytes in the capillaries. The McManus periodic acid-leukofuchsin and the Hale stains for complex polysaccharides revealed the glomerular basement membranes of the experimental animals to be thickened. While often the thin sharply outlined membrane could still be recognized in the periodic acid stain, a diffuse less dense material was found on both the endothelial and epithelial sides of the membrane. The membrane occasion-

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TABLE I. Chemical Analysis of Skin. (Per 100 grams of dry fat-free solids.)

Group and No. rats		H_2O (ml)	Na (mM)	K (mM)	Cl (mM)	Collagen nitrogen (g)
Control	Mean	267.5	27.2	21.0	23.7	6.59
(6)	S.E.	4.1	.5	1.3	2.4	.12
Control and	Mean	250.7	26.3	18.1	21.7	6.05
NaCl load	S.E.	4.1	1.6	1.2	.9	.09
(11)						
Edematous	Mean	475.9	58.5	19.3	48.9	6.68
(6)	S.E.	11.4	5.5	.5	4.0	.34
Edematous and	Mean	534.5	73.4	17.6	59.5	6.31
NaCl load	S.E.	50.8	6.7	.6	5.6	.30
(12)						

ally appeared duplicated. With the Hale stain for acid mucopolysaccharide, the membrane was less clearly defined and had the appearance of a diffuse thickening. No increase in the number of either the epithelial or endothelial nuclei of the glomeruli was recognized. The glomerular capillaries occasionally contained lipid-laden endothelial cells with Sudan black stain; these cell bodies also stained positively with the McManus technics. The convoluted portions of the renal tubules contained a few acellular casts, especially in their more distal portions, and in these areas the tubular epithelium was compressed, becoming thin and elongate. The remainder of the convoluted tubular epithelium was swollen and pale in routine stains (toluidine blue eosin). Mitochondrial stains (Mallory's phosphotungstic acid hematoxylin technic) showed a marked loss of these elements, with the concomitant appearance of small fat droplets in fat stains (Sudan black) in the infranuclear portion of the cell, away from the luminal surface. These small droplets were of such a size as not to appear as vacuoles in routinely stained paraffin-embedded sections.

In the livers of the experimental animals there were very small variable amounts of fat in the liver cells at the periphery of the lobules. The lipid droplets were small and did not displace the parenchymal nuclei. The liver cells had an increased and abnormal number of mitotic figures. The parenchymal-cell nuclei were large, with prominent nucleoli. The Kupffer's cells were lipid-laden and like the glomerular endothelial cells had sharply positive periodic acid-leukofuchsin granules. The sections of skin and of the edematous omentum were, in paraffin-embedded sections, not significantly different from the controls. No histological differences were demonstrated between the salt-loaded and non-salt-loaded experimental animals.

The changes in tubules and glomeruli are reversible in short-term experiments [5 to 20 days (unpublished observations)]. Unlike the renal damage associated with the injection of anti-rat-kidney serums, no proliferation or collagenization of the glomerulus ensues(6). The absence of proliferative glomerular lesions, as well as the absence of tubular necrosis, distinguishes this lesion from poisoning with heavy metals, cantharidin or diphtheria toxin(7).

In the edematous rats, the mean total nitrogen of serum corrected for water was 5.75 ± 0.20 (S.E.) g per liter, and the mean nonprotein nitrogen was 1.119 ± 0.06 (S.E.) g per liter. In the control animals, the mean total nitrogen of serum corrected for water was 7.79 ± 0.32 (S.E.) g per liter, and the mean nonprotein nitrogen was 0.43 ± 0.02 (S.E.) g per liter.

The water, electrolyte, nitrogen and collagen contents of the tissues have been expressed, using dry fat-free solids as a reference base. The skin of edematous rats contained a subdermal layer of gelatinous material several millimeters thick. This layer gradually liquefied after a few minutes at room temperature and formed a pool of water which immersed the skins in their weighing bottles. Values for the entire skin are indicated in Table I. As shown by

Heringa and Weidinger, the skin of young rats appears to contain more water and less collagen than does that of older rats(8), dogs, or human beings(9). These water, Na, and Cl contents of the edematous skins were approximately twice those of the controls. The difference was exaggerated in the NaCl-loaded, edematous animals. Skin of NaCl-loaded controls contained less H_2O, Na, and Cl than did that of the non-loaded animals.

Some features of the chemical composition of muscle samples in terms of non-collagenous dry fat-free solids are indicated in Table II. The increment of H_2O in muscle in the edematous groups was less striking than was the case for skin. Relative changes in Na and Cl content seemed proportional to those observed in skin. The potassium content of the muscles was slightly reduced during edema, particularly following NaCl loading. Non-collagenous nitrogen was unchanged, but the collagen content per unit of cell mass was increased in the loaded hypoproteinemia edematous rats, suggesting a loss of muscle sarcoplasm with replacement by collagen. Derived data for muscle, based on the premise of an entirely extracellular position for chloride, and analytic data for extracellular fluid composition also are indicated in Table II. NaCl loading did not alter the intracellular or extracellular contents of H_2O and K in control muscle. Slight reduction of intracellular sodium content in the loaded control animals was not significant. Edema fluid in the experimental rats was largely extracellular. Intracellular K content was not significantly decreased during edema. The calculated concentration of K in the intracellular water was significantly reduced in the NaCl-loaded edematous rats. This reduction reflected intracellular diffusion of water, and the cation concentration deficit was partially compensated by increased intracellular Na concentration.

The relative distribution of edema in skin and residual carcass (all muscle, skeleton, and viscera) referred to the actual content of dry fat-free solids of the pair-fed control may be assessed by reference to Table III. More than half of the excess tissue water, Na, and Cl content of the edematous rats was found in skin and subcutaneous tissues. Of the to-

TABLE II. Chemical Composition of Muscle in Terms of Non-Collagenous, Dry, Fat-Free Solids.

Group and No. rats		Extracellular								Intracellular					
		Per 100 g non-collagenous, dry, fat-free solids					Per liter H_2O			Per 100 g non-collagenous, dry, fat-free solids				Per liter H_2O	
		H_2O (ml)	Na (mM)	K (mM)	Cl (mM)	Collagen (g)	Na (mM)	K (mM)	Cl (mM)	H_2O (ml)	Na (mM)	K (mM)	Non-collagen (g)	Na (mM)	K (mM)
Control (6)	Mean	53.9*	7.7*	.23	6.77*	7.30	143.3	4.31	125.8	314.3	6.3	50.8	14.27	20.1	161.7
	S.E.	.8	.1	.02	.09	.24	2.6	.28	1.1	3.1	.3	.5	.11	1.0	2.9
Control and NaCl (8)†	Mean	53.8*	7.8*	.21	6.69*	7.36*	144.4	3.89	122.3	315.3*	5.1	50.2	13.96	16.1*	159.2*
	S.E.	1.1	.2	.02	.12	.40	1.2	.24	1.5	2.3	0.7	.6	.15	.8	2.6
Edematous (6)	Mean	106.4*	14.9*	.51	12.75*	7.80	139.6	4.74	120.0	327.9	9.1	50.5	14.25	27.4	153.8
	S.E.	6.1	1.0	.06	.70	.56	2.7	.42	1.7	6.8	2.8	1.5	.22	8.1	2.4
Edematous and NaCl (9)†	Mean	136.9*	19.4*	.53	16.83*	10.59*	142.1	4.01	123.2	337.6*	8.1	48.6	14.05	23.5*	144.4*
	S.E.	6.8	.9	.03	.77	.64	1.8	.33	1.2	7.6	1.3	.7	.34	3.1	3.5

* Statistically significant differences ($t-p<0.05$) between means of experimental and appropriate control groups.

† Insufficient material to determine collagen, non-collagenous nitrogen, or Cl serum accounts for number of animals represented. Data of other 3 and 4 muscle samples were within S.E. of mean group per 100 g dry fat-free solids.

TABLE III. Entire Skin and Carcass.*

	Dry, fat-free solids (g)	Total nitrogen (g)	Collagenous nitrogen (g)	H_2O (ml)	Na (mM)	K (mM)	Cl (mM)
			Controls				
Carcass	15.92 ± .51	1.82 ± .06	.42 ± .03	47.98 ± 2.15	3.35 ± .13	5.38 ± .14	2.30 ± .01
Skin	3.43 ± .10	—	.22 ± .01	8.98 ± .32	.94 ± .04	.73 ± .01	.80 ± .03
Total	19.35	—	.64	56.96	4.29	6.11	3.10
			Edematous Corrected to dry, fat-free solids content of pair-fed control				
Carcass	(14.86 ± .40)†	1.82 ± .28	.44 ± .03	55.05 ± 2.68	4.20 ± .15	5.12 ± .15	2.93 ± .17
Skin	(2.91 ± .08)	—	.22 ± .001	16.21 ± 1.47	2.18 ± .20	.64 ± .001	1.74 ± .15
Ascites	—			21.7	3.32	.11	2.54
Total	(17.77)	—	.66	92.96	9.70	5.87	7.21

* Mean and stand. error of mean compiled from 6 rats in each group. Three rats in each group received an additional NaCl load P.O. (*cf.* text).

† Numbers in parentheses refer to observed dry fat-free solids of the experimental animals.

tal accumulation of edema fluid, ½ was ascitic fluid, slightly more than ¼ in skin, and slightly less than ¼ in residual carcass. Total body K appeared to be slightly decreased by edema, particularly following an additional NaCl load.

Discussion. It is evident that injection of this aminonucleoside into immature male rats provides a convenient tool for exploring many features of a renal disease with manifestations similar to those of the nephrotic syndrome in children. Edema fluid accumulates largely as free fluid in the peritoneal cavity and as a gel in skin—presumably dissimilar extracellular reservoirs. The residual carcass—including the entire muscle mass, in which there was insignificant loss of intracellular K, and skeleton—is a lesser participant in the generalized edema. Similar conclusions were reached by Klose, following analysis of 2 edematous infants(10).

In skin, apparently the major tissue reservoir for edema, the "sodium space" usually exceeded the "chloride space" in these young rats, in contrast to the data obtained in older rats(11). Na/Cl ratios of skin were slightly greater than those in serum water. Whether all or only a portion of the bodily excess of the Na and Cl accumulated in edematous skin is freely exchangeable with the remainder of Na and Cl in the ascitic and interstitial fluids, is being determined.

Summary. The nephrotic syndrome was regularly induced in immature male rats by 12 daily injections of an aminonucleoside. The essential renal lesions were thickening of the glomerular basement membranes and loss of mitochondria in renal convoluted tubular cells. Electrolyte composition of serum, ascitic fluid, muscle, entire skin, and carcass also was determined. Suitable corrections for collagen were used where applicable. Skin was shown to be the major tissue reservoir for accumulation of edema fluid.

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Mechanism of Alcoholic Stimulation of Gastric Secretion.* (21834)

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The general pharmacological action of alcohol on the body is one of depression. The major exception to this rule is its action on the stomach. Secretion of acid gastric juice is stimulated by either oral or parenteral alcohol. Introduction of alcohol into the stomach or intestine of anesthetized dogs stimulates the secretion of acid gastric juice(1). Ten per cent alcohol instilled in a Thiry's fistula of the duodenum and jejunum stimulates secretion of the acid gastric juice from a Pavlov pouch dog(2). Division of the vagus nerves supplying a Pavlov pouch has little or no effect upon the secretory response to alcohol (3). It has been shown that alcohol liberates histamine from living tissue(4), and it has been postulated that this might represent the method by which gastric secretion is stimulated.

Method. 1) Two Heidenhain pouches were constructed from the body and fundus of the stomach of one dog (Fig. 1). These pouches were completely separated from the main stomach and from each other and were drained to the exterior with a stainless steel cannula in each(5). Following recovery from

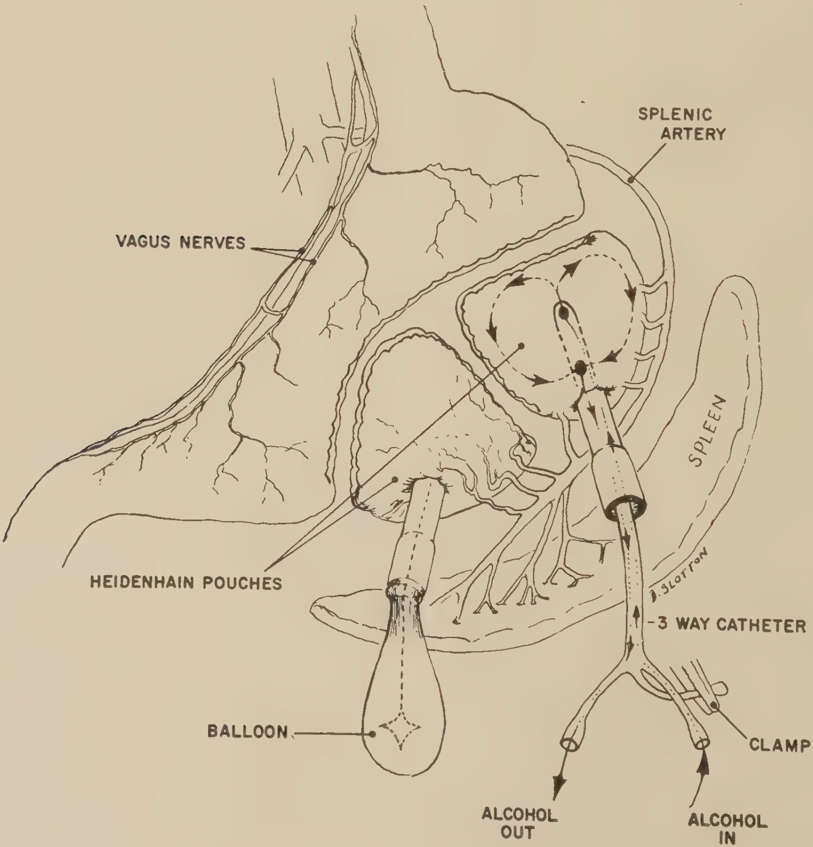

FIG. 1. Dog prepared with 2 separate isolated fundic (Heidenhain) pouches.

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surgery, the animal was maintained chronically in good nutritional and electrolyte balance. In the fasting animal a small test balloon was attached to one cannula for collection of gastric juice. A 3-way Foley bag catheter was introduced into the other pouch via the second cannula, the bag inflated and 5 or 10% alcohol solution perfused through the pouch.

2) In 6 dogs large Heidenhain pouches of the body and fundus of the stomach were prepared. The 3-way Foley catheter was introduced as described above, while the animal was in the basal secretory state. Solutions of alcohol were perfused through the single Heidenhain pouch and the perfusates collected for four 30 minute periods. The pH of the perfusate was determined, and the HCl content measured by titration back to the original pH of the alcohol solution (6.1). Gastric juice secreted by the pouch was collected for one hour following the perfusion.

3) In another dog a Heidenhain pouch was prepared, and at a secondary operative procedure (Fig. 2) the antrum of the stomach separated from the main stomach by a double layer mucosa barrier as previously described(6). The duodenum was transected immediately beyond the pylorus and the proximal end brought to the skin as a cutaneous fistula. The distal duodenum was anastamosed to the body of the stomach. A cannula was introduced into the main stomach and brought to the exterior as a gastric fistula. Following recovery of the animal, gastric juice was collected from the Heidenhain pouch, while solutions of alcohol were either introduced into the main stomach through the gastric cannula or perfused through the antrum via the duodenal fistula.

Results. In the animal prepared with 2 Heidenhain pouches (Fig. 1) perfusion of one pouch with 5% alcohol over a 2½-hour period produced no secretion of gastric juice from the other. Following perfusion there was no acid secreted by the perfused pouch. The experiment was repeated with 10% alcohol for the same period with the same negative result. Intravenous infusion of 250 cc of 5% alcohol resulted in secretion of acid gastric juice by both pouches, which was about half of that following 1 mg of histamine phosphate given subcutaneously.

TABLE I.

	Minimum pH of perfusate	HCl/2 hr (mEq)	Secretion after perfusion (mEq/hr)
Dog 1	7.04	0	0
	6.79	0	0
	6.52	0	0
	5.68	.007	0
	4.78	.01	0
	4.77	.015	0
	4.31	.03	0
	3.87	.13	0
	3.61	.22	0
	2.83	1.51	0
	2.78	2.19	0
	2.12	.65	0
2	6.86	0	0
	6.74	0	0
3	4.09	.09	0
	2.19	5.40	0
4	3.10	.06	0
	2.62	4.75	.013
5	3.41	0.3	.33
	2.51	2.95	0
	2.23	11.0	0
	2.20	16.4	0
	2.08	11.8	0
	1.95	18.6	.48
	1.93	26.5	0
6	3.51	0.26	0
	3.43	1.8	0
	2.83	1.64	0
	2.18	19.25	0

In the 6 animals prepared with single Heidenhain pouches direct perfusion of the pouches with 5% alcohol was done on 29 occasions (Table I).

Subcutaneous histamine and oral or intravenous alcohol strongly stimulated all Heidenhain pouches to secrete acid gastric juice. Perfusion of the Heidenhain pouches with physiological saline resulted in no HCl secretion in 2 tests. With distilled water as a perfusate significant amounts of HCl were secreted in 2 of 4 studies.

Perfusion of the isolated antrum (Fig. 2) with 250 cc of 5% alcohol was followed in 10-30 minutes by a copious secretion of highly acid gastric juice from the Heidenhain pouch. In 6 tests the output of free HCl during the response varied from 1.89 to 6.21 mEq, averaging 4.24 mEq. An example is presented in Table II. Perfusion with 10% alcohol caused

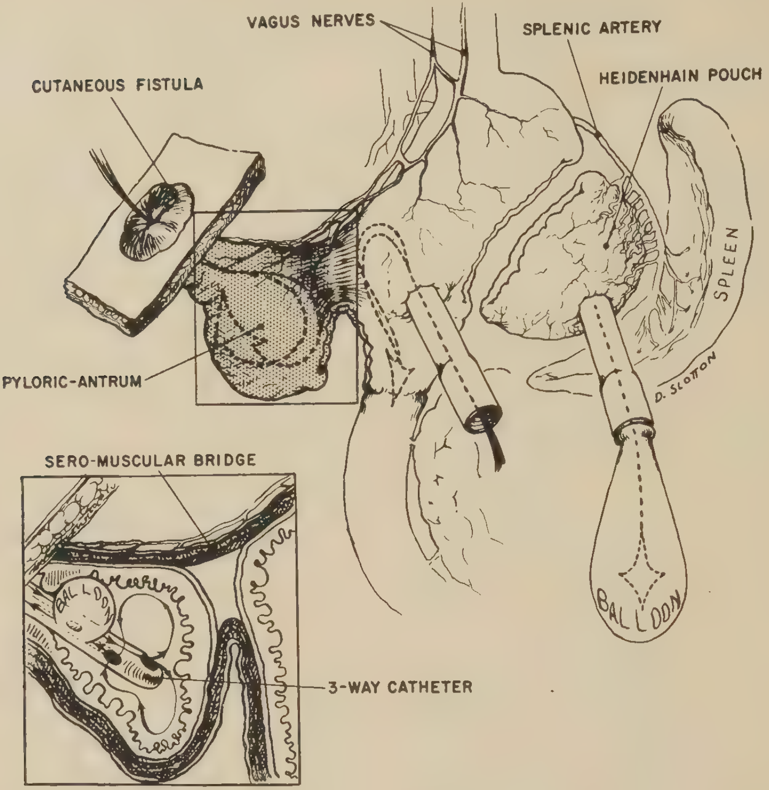

FIG. 2. Dog prepared with Heidenhain pouch, gastric fistula, and isolated pyloric antrum.

a somewhat stronger response, 6.24 mEq. When the pH of the alcoholic perfusate was adjusted to 1.2 no response was elicited from the Heidenhain pouch in 2 tests. Perfusion of the isolated antrum with crude liver extract (10%) resulted in secretion from the Heidenhain pouch about equal to that following 5% alcohol.

TABLE II. Perfusion of Isolated Antrum with Dilute Alcohol (Fig. 2).

	Time	Vol	Free acid (mEq/l)
Collections of gastric juice from Heidenhain pouch	11:30–12	0	—
Antral perfusion with 5% alcohol begun at 12	12:30	0	—
	1	3.5	105
	1:30	8.0	136
	2	9.5	140
	2:30	7.5	139
Perfusion ended; 250 cc total	3	5.5	139
	3:30	2.5	122
	4	2.5	114
	Total output	5.20 mEq free HCl	

In the same animal injection of alcohol directly into the main stomach also resulted in a good secretory response by the Heidenhain pouch, but considerably less than that following antral perfusion. In 6 tests the response lasted 3-4½ hours and produced 0.60 to 5.33 mEq of free HCl, averaging 2.40 mEq. An equal volume of 10% alcohol produced 4.79 mEq. Acidification of the alcohol to pH 1.2 had no inhibitory effect on this response in 2 tests.

Discussion. Perfusion of a Heidenhain pouch with 5% or 10% alcohol for prolonged periods failed to elicit any secretory response from a second separate fundic pouch in the same animal. If contact liberation of histamine were responsible for the stimulating effect of alcohol, one would expect histamine via the blood stream to stimulate the second pouch. Intravenous alcohol was shown to stimulate both pouches as did subcutaneous histamine.

In the second experiment (Table I) there was usually no significant secretion of hydrochloric acid from Heidenhain pouches perfused directly with 5% alcohol. In 5 of the animals less than 1 mEq of titratable HCl was secreted in the 2-hour period during 15 of 22 perfusions. In the sixth animal, (#5), secretion of HCl as much as 26.5 mEq in 2 hours was seen during perfusion. Histological examination of the pouch in this animal disclosed a large remnant of antrum inadvertently incorporated in the distal angle of the pouch. From these data it would appear that direct application of dilute alcohol to the acid secreting mucous membrane produces no or very slight secretion of HCl.

In the third experiment perfusion of the isolated pyloric antrum with 5% alcohol solution produced a copious secretion of highly acid gastric juice from the Heidenhain pouch. This response compared favorably with that obtained from crude liver extract. When the pH of the alcohol was reduced to 1.2, perfusion of the antrum failed to elicit a secretory response from the Heidenhain pouch. This effect of low pH is a known characteristic of the "gastrin" mechanism(6). Therefore, alcohol, like many foodstuffs, is a chemical stimulant for the gastric phase of gastric secretion, an endocrine function of the pyloric antrum.

Alcohol infused into the main stomach of the animal used in Exp. 3 (Fig. 2) also strongly stimulated the secretion of acid gastric juice by the fundic pouch. Since the antrum was isolated and the acid secreting mucosa seemingly only weakly stimulated by alcohol if at all, this response is presumably due to the intestinal phase of gastric secretion. The secretory effect observed is only about one-half as strong as that following antral perfusion. The antral perfusate escaped to the exterior so that presumably absorption was less than that after intragastric instillation of alcohol. No alcohol blood levels were determined however. It is also interesting to note that acid alcohol was effective when introduced directly into the stomach in sharp contrast to its failure in the antrum. This raises the question as to whether or not the hormonal agent of the intestinal phase is different from "gastrin."

From the above data it would appear that about ⅔ of the acid gastric juice secreted in response to ingested alcohol results from stimulation of the antral "gastrin" mechanism, and approximately ⅓ from intestinal stimulation. The effect of alcohol on the nervous phase of gastric secretion has not been studied in these experiments.

Conclusions. 1) Dilute alcohol applied directly to the mucosa of the body and the fundus of the stomach does not usually stimulate secretion of acid gastric juice. 2) Dilute alcohol is a powerful stimulant of the "gastrin" mechanism of the pyloric antrum. It appears that in response to ingested alcohol, ⅔ of the gastric juice secreted results from this mechanism. 3) Alcohol is known to activate the intestinal phase of gastric secretion. This mechanism accounts for about ⅓ of the secretory response to ingested alcohol.

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Presence of Volatile Fatty Acids in Bovine Semen.*† (21835)

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In the course of an investigation of the dissimilation of uniformly labeled glucose by bovine spermatozoa, some of the radioactivity was recovered in the acid steam distillate(1). A search of the literature revealed no reports of the determination of volatile acids in semen, although acetate has been postulated as a product of pyruvate catabolism in bovine semen(2,3). The experiments reported herein were undertaken to determine to what extent volatile acids occur in freshly ejaculated bovine semen, and to determine the relative amounts of the volatile acids present.

Material and methods. Semen was collected from Holstein and Guernsey bulls with the artificial vagina. Individual ejaculates were deproteinized with H_3PO_4(4) at the time interval after ejaculation set for each particular experiment. After addition of H_3PO_4, ejaculates were pooled; each of the 8 experiments involved a total of 12-45 ml of semen from 4-9 bulls. The H_3PO_4 treated semen was adjusted to pH 9.0 with KOH, filtered, and the volume adjusted to 50 ml. The filtrate was steam distilled after adjustment to pH 3.0 with 10 N H_2SO_4(4). On completion of distillation, CO_2 was purged from the distillate and the distillate titrated with standardized NaOH to determine the total volatile acids present. To separate the component acids the alkalinized distillate was evaporated to dryness and chromatographed on silica gel(5).

Results. The concentration of total volatile acids and relative amounts of the major components in each of the experiments are shown in Table I. Although the relative amounts of individual acids varied from trial to trial, formic acid, acetic acid and small amounts of other volatile acidic substances were detected. A typical profile of elution of the acids from the silica gel column is shown in Fig. 1. Elution patterns were compared to those of known acids; in addition the fractions comprising each major peak were combined, reduced in volume and checked by paper chromatography of the diethylamine salts (6). Since paper chromatography does not provide adequate resolution of formic and acetic acids, aliquots of the fractions from the silica gel column tentatively identified as formic and acetic acids were oxidized with HgO to remove formic acid(7). The residual solution in each case was steam distilled and rechromatographed on silica gel. The selective oxidation procedure verified the position of the formic acid peak eluted from the column.

TABLE I. Volatile Fatty Acids Present in Pooled Ejaculates of Bovine Semen.

Exp. No.	Age of semen (min.)	Volatile acids (meq/100 ml)	% volatile acids recovered as		
			Formic	Acetic	Other
1	120	2.50	Not determined		
2	5	2.34	24	57	19
3	120	1.78	44	56	0
4	30	2.51	22	72	6
5	30	1.62	Not determined		
6	180	1.56	30	66	4
7	30	1.08	70	15	15
8	5	.79	20	78	2

Small peaks were observed in Exp. 2 and 7 which corresponded to the expected locations of propionic and butyric acids. However, the amount of material was so limited that the presence of these acids could not be verified by paper chromatography. Peaks similar in location to the one represented by fractions 36-38 in Fig. 1 were observed in Exp. 2, 4, 6, 7 and 8, but this substance has not been identified. When paper chromatographed, this substance yielded an R_f of 0.48, compared to 0.24, 0.26, 0.39 and 0.60 for standards of formic, acetic, propionic and butyric acids, respectively.

While it has been possible to demonstrate the occurrence of formic and acetic acids in

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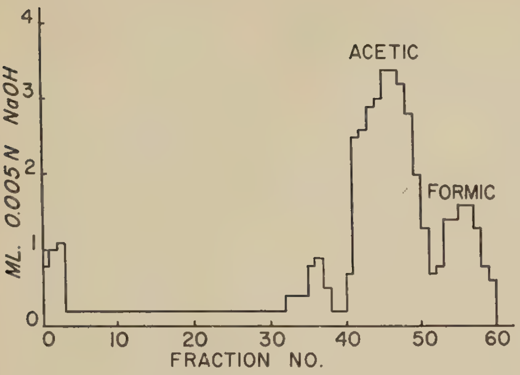

FIG. 1. Elution profile of volatile fatty acids (Exp. 4) from a silica gel column developed by the addition in turn of 150 ml each of 0, 1, 5 and 10% n-butanol in benzene. Fractions were 10 ml each.

freshly ejaculated bovine semen, the significance of these substances to the cell has not been determined. Melrose and Terner(2) have suggested that spermatozoa form 1 M each of lactic acid, acetic acid and CO_2 from 2 M of pyruvic acid, while Tosic(3) has proposed that pyruvic acid and H_2O_2 react to form acetic acid, CO_2 and H_2O. Either reaction could account for the presence of acetic acid, which subsequently may be oxidized as a source of energy by the spermatozoa(8).

The results in Table I reveal no consistent trend with age of semen in either total volatile acids or relative amounts of individual acids. As a check on this observation trials were conducted in which each ejaculate was split and one moiety deproteinized 5 min. after collection while the other was incubated 180 min. at 20°C before being deproteinized. No consistent difference was observed in total or constituent acids between the 5- and 180-min. moieties. Thus in undiluted semen these acids appear to be either in catabolic-anabolic equilibrium or relatively inert metabolically.

Summary. Concentrations of 0.79-2.50 meq of volatile fatty acids per 100 ml were found in pooled ejaculates of bovine semen. Acetic and formic acids were identified as the major volatile fatty acid constituents by column chromatography, paper chromatography and selective oxidation of the formic acid.

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Infection of Chicks by Intranasal Route with Rous Sarcoma Virus.[*] (21836)

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Droplet infection is a major method of spread in many virus infections. Reports by Burmester and Gentry(1,2) indicate that the virus of visceral lymphomatosis may be spread in this manner. In an attempt to determine the natural mode of infection of chickens by the Rous sarcoma virus, it was of interest to see if chicks could be infected by intranasal inoculation. It was also important to determine success of this manner of transfer since this procedure is probably associated with little or no initial host cell injury. Some investigators(3) have stated that initial trauma of tissue is essential before sarcomas can develop as a result of Rous virus infection. Finally if the intranasal route

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TABLE I. Inoculation of Chicks Intranasally with Rous Sarcoma Virus.

Dose, ml*	Age at inoculation	No. observed	No. with tumors	Time of observations after inoculation	Location of sarcomas
1.1	2 days	10	6	8 to 109 days	4 chicks lungs only; 1 lungs and heart; 1 generalized
Aerosol	1 day	4† 4‡	0 0	3 mo to 3 yr	No gross evidence of Rous infection
.5	1 wk	3	3	23 to 48 days	1 chick lungs only; 2 generalized
1	2 ”	4	2	24 and 52 days	1 chick lungs only; 1 generalized
2	3 to 4 wk	5	3	20 to 36 days	1 chick lungs only; 2 generalized
3	5 wk	10	4	31 to 56 days	2 chicks lungs only; 1 lungs and heart; 1 generalized

* See text for method of inoculation.
† Received methylcholantherene in sesame oil 31 days after exposure to virus.
‡ Received sesame oil without methylcholantherene 31 days after exposure to virus.

proved successful, it was planned to observe the infected birds over a long period of time in order to determine if infection with a carcinogenic virus in early life might result in cancer in adulthood(4).

Materials and methods. The Rous tumors used were 5 to 8 days old when harvested. These growths were stored at –30°C for 1 to 2 months before being doubly extracted in sterile saline, preparatory to passage of the extracts through #545 celite columns(5,6). The extract, a one to 20 dilution by weight of the tumor tissue, having been passed through the celite, the column was then developed with physiological saline, and eluted with distilled water. Active eluates from celite adsorption columns were used in all experiments.

Intranasal inoculation of these eluates was accomplished in one of 3 ways: 1) by placing 0.1 ml of the virus solution on the external nares of sodium nembutal anesthetized chicks and allowing the material to be inhaled; 2) by placing a short piece of 10 mm glass tubing over the beak and nares of unanesthetized birds and adding slowly active eluate to the amount desired; 3) by spraying the Rous virus from an atomizer into a sealed chamber into which chicks were placed.

White Rock chickens were used, their ages varying, depending upon the experiment, from one-day-old chicks to 5-week-old birds. Following inoculation all animals were observed under laboratory conditions until their size necessitated removal to the country where range conditions existed. Duration of observations extended up to 3 years.

Results. In the first experiment of this series intranasal inoculations were accomplished in a manner described in method 1 above and 8 days later the same chicks received a second intranasal inoculation by method 2, using a volume of 1.0 ml. Of the 10 birds inoculated 6 developed fibro-sarcomas and 5 of these birds were dead or dying of their growths in from 8 to 23 days after infection. The sixth bird died of sarcomatosis 109 days after the last inoculation (Table I). In the early deaths typical Rous tumors filled both lungs and one of the birds had in addition sarcomas in the myocardium. The other organs were grossly negative. In the bird dying later, tumors were present in the lungs, adrenals, liver, and in the heart. The 4 birds that did not develop growths were observed from 200 days to 3 years after inoculation before autopsies were done.

It was evident from the above experiment that the intranasal route can be used as a means of infecting chicks with Rous virus. Another group of chicks was inoculated by Rous virus aerosols. In order to check the effect of the aerosol state on the activity of the virus, active eluates were sprayed from an atomizer into a 3 liter beaker covered with aluminum foil. The condensate collected at the bottom of the container was inoculated intramuscularly into the left breast of 5 young chicks. Within 8 days typical Rous sarcomas developed in all birds.

The aerosol environment not being deleterious to the Rous agent, the next experiment in this series was undertaken. Ten one-day-old chicks were exposed to 20 ml of nebulized Rous virus for 5 minutes in a sealed container every other day for 5 times. Each time the atmosphere in the container was evacuated for 5 minutes with water-pump suction before the chicks were removed. There was no evidence of an accumulation of eluate on the walls or bottom of the container. At the end of 31 days after the last exposure the birds showed no external evidence of Rous infection and the 10 birds were then divided into 2 groups of 5 each. Into birds of one group 1 mg of methylcholantherene in sesame oil was inoculated intramuscularly into the left breast while into the birds of the other group 0.1 ml of sesame oil alone was injected in the same manner. In addition 4 birds of comparable age who had not received virus were given methylcholantherene by the same technic. All of these animals were observed for various periods of time up to 3 years. None of the chickens receiving the aerosol and sesame oil with or without carcinogen developed growths (Table I). Two birds, one in each group were unaccounted for at the end of the experiment. Those receiving methylcholantherene and no virus lived 2 years, at which time they were sacrificed. One bird showed no lesions and another a necrotic, cystic tumor of the kidney which did not have the histological characteristics of the Rous sarcoma. Observations were not continued on the other 2 birds of this group.

The final experiment in this study involved the intranasal inoculation of birds of different ages. Three groups of 5 individuals each were arranged according to age: 1, 2, and 3 to 4 weeks respectively. Each age group was inoculated by method 2 above with increasing amounts of virus corresponding to age of the birds: 0.5 ml, 1.0 ml, and 2.0 ml. Ten 5-week-old chicks constituted a fourth group, these being inoculated in a similar manner with 3.0 ml of virus.

Of the birds one-week-old at the time of inoculation, observations were possible on 3, two being unaccounted for. One autopsied 48 days after infection showed sarcomas limited to its lungs, while the 2 other birds had sarcomatosis as shown by autopsy on the 23rd and 40th days respectively (Table I). It is of interest that one of these showed growths which occurred in organs not usually involved in Rous virus infection, suggesting possible alteration of specificity of the agent(7,8). There was a sarcoma of the bony pelvis, hemorrhagic growth on the sternum, 2 tumorous ulcers in the mucosa of the gizzard as well as growths deep in the gizzard musculature; also scattered sarcomas throughout the proventricules were present (Fig. 1). The myocardium was almost completely replaced by tumor. While frank hemorrhagic disease(9) was not observed, evidence of a hemorrhagic diathesis was suggested by the blood streaking of the muscles in the breast and leg.

In the 2-week-old group 2 birds died in 24 and 52 days after inoculation, one with lung sarcomas and the other with tumors in the lungs, liver, spleen and heart (Table I). Two showed no evidence of Rous infection; one of these died after 11 months observation, the other after 20 months, the latter showing extensive peritonitis. There were no observations on the fifth bird.

Three birds of the 3- to 4-week-old group died between 20 to 36 days after infection. Two of these had extensive sarcomatosis involving many organs and tissues, including lungs, with extension to the thoracic wall and mediastinum. The great vessels, oesophagus, trachea, myocardium, and omentum were also involved. The third bird had tumors limited only to the lungs (Table I). The 2 chickens showing no Rous sarcomas were observed for 12 months and 3 years respectively. The latter bird at the time of sacrifice had a small lesion on the surface of the liver. Transfer of this lesion to young chicks gave no evidence of Rous virus infection.

Birds in the 5-week-old group were observed up to 3 years. Six of these were negative for Rous infection within a 5-month to 3-year period. Four died of their growths 31

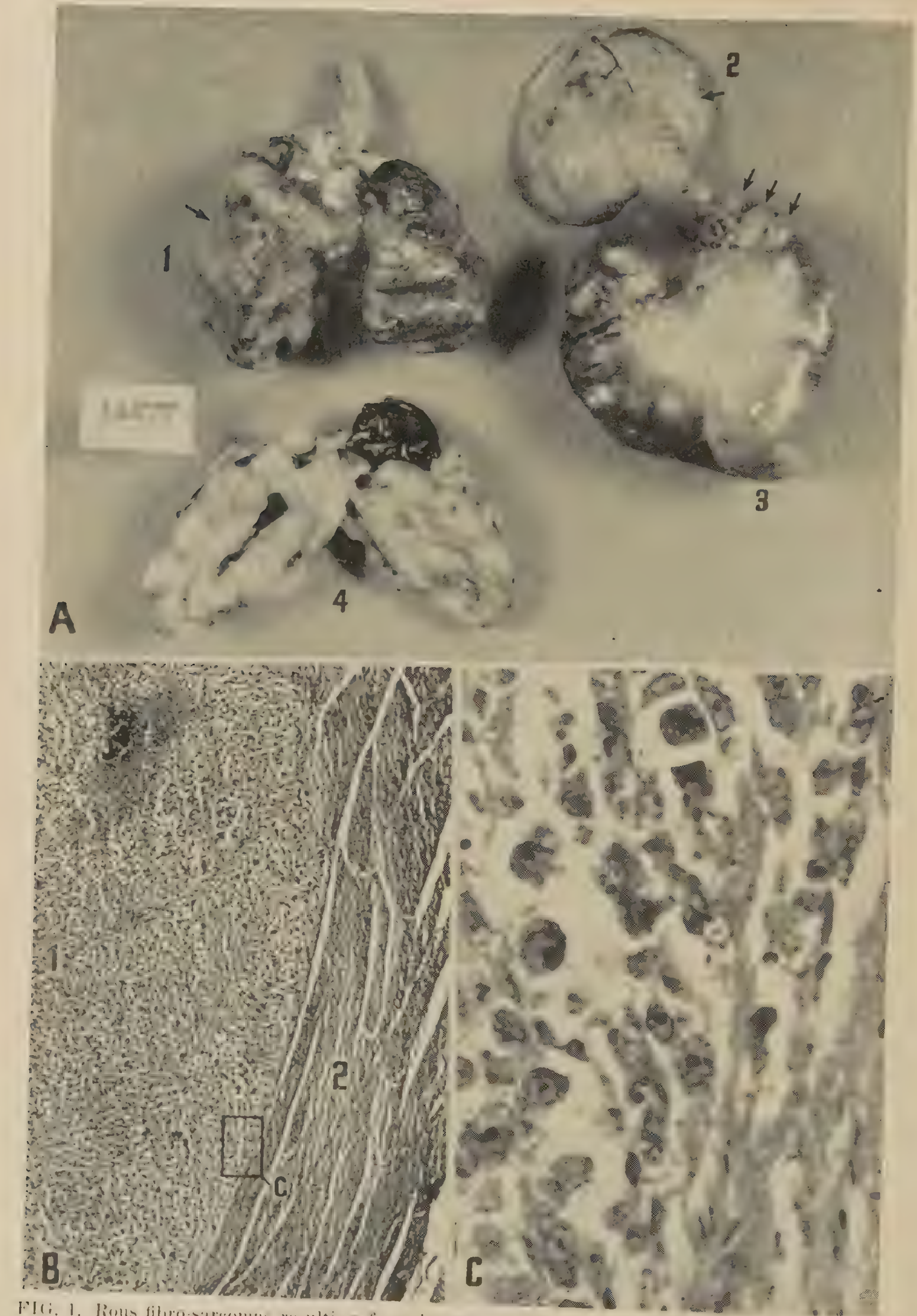

FIG. 1. Rous fibro-sarcomas resulting from intranasal infection of chick. A *Gross lesions*: 1, lungs; 2, proventriculus; 3, gizzard; 4, heart. Arrows point to tumors. The arrow in A1 points to lobe almost completely replaced by tumors. B *Microscopic lesions*: 1, tumor; 2, muscle of gizzard. Mag. 55×. C Insert of B. Mag. 708×.

to 56 days after infection. In 2 birds sarcomas were limited to the lungs; in one in addition to the lungs, the right myocardium was involved. In the fourth extensive disease was present in the lungs, mediastinum and gizzard (Table I).

Discussion. Little or nothing is known of the manner of spread under natural conditions of Rous virus infection in chickens. Since transmission by inhalation of virus-contaminated droplets is an important means of spread of many virus diseases and the virus of visceral lymphomatosis may possibly be naturally spread by this means(1,2), it was of interest to determine if the Rous virus could be infective by the upper respiratory route. Birds were successfully infected by intranasal inoculation of the agent when the virus solution was placed directly over the external nares of the bird and allowed to be drawn in on inspiration. As an aerosol the agent did not produce growths. That this might be the result of dose rather than the effect of the aerosol state on the virus was shown by the fact that chicks inoculated intramuscularly with virus aerosol condensates developed tumors. Burmester and Gentry (1) found that aerosol inhalation of visceral lymphomatosis virus was not as efficient in infecting chickens as other means.

It is significant that although the lesions in birds inoculated with Rous virus by the intranasal route centered primarily in the lungs, 9 of the 18 birds showing sarcomas had growths in other organs in addition, and 7 of these had extensive sarcomatosis. The heart was the organ involved next in frequency to the lung. No birds were observed that possessed growths in organs and tissues without tumors in the lungs when the intranasal route was used. In some individuals fibro-sarcomas were present in bone, adrenals, proventiculus, and deep in the muscle of the gizzard. One bird showed tumorous ulcerations of the gizzard mucosa as previously described by Shrigley(8). The growths associated with the gastro-intestinal tract were considered not to be evidence of initial infection by this route since it is known that chicks may eat Rous tumor without developing growths.

Some investigators(3) have expressed the idea that before the Rous agent can produce initial tumors, injury to the tissue must take place. The present experiments on intranasal inoculation would suggest that trauma is not a conspicuous feature of infection. It is conceivable that inhalation of the virus solution together with the mild struggling of the bird might alter the integrity of the mucous membrane of the upper respiratory tract, but this possibility seems remote. As infection was obtained with 0.5 ml of inoculum, the volume of inoculum likewise would appear unimportant in this connection.

Finally there seems to be a definite effect of age on the success of infection by this route. In birds 5 weeks of age at the time of infection only 40% developed growths even though a relatively large amount of inoculum was used.

Duran-Reynals(4) has suggested that cancer may be an expression of a virus infection acquired in childhood, harbored throughout life only to become manifest in adulthood as malignant growths. Carr(10) has reported a latent period of this nature in chickens for Rous virus. For this reason young birds inoculated in this study were observed for a period in some cases up to 3 years. If the intranasal route is the natural means of spread for the Rous agent it was thought that these experiments would afford an ideal setup to observe this latent action of the virus. The data here however suggest that if a bird is to show evidence of Rous infection at all it will do so some time within 3½ months after the intranasal inoculation.

Summary and conclusions. 1. It is possible to infect chicks with the Rous sarcoma virus by using the intranasal route. 2. By this route, the lungs always became involved if infection took place, but growths were by no means limited to this organ. 3. Widespread involvement of organs and tissues may result from inoculation by this route. 4. Inhalation of aerosol preparations of the Rous virus did not yield recognizable Rous virus infection in this study. 5. The organs and tissues involved with sarcoma in some instances were those not usually associated with Rous infection when other routes of inoculation are used. 6. No hemorrhagic disease was observed in infected chicks. 7. The possible relationship of the intranasal route to the method of spread of natural Rous virus infection is considered as is also the bearing of these experiments on cancer in general.

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Detroit-6 Strain of Human Epithelial-Like Cells: Virus Susceptibilities.* (21837)

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(Introduced by Gordon H. Scott.)
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A new strain of human epithelial-like cells derived from the bone marrow of a patient with carcinoma of the lung has been described by Berman, Stulberg, and Ruddle(1). For reasons discussed it was presumed that these cells might have originated from metastatic carcinoma cells in the marrow. Since its isolation in November, 1954, this strain has been maintained through continuous subculture on glass without discernible change in morphologic characteristics or growth pattern. These cells have been designated the Detroit-6 strain.

We have now established that the Detroit-6 strain fulfills certain criteria(2) for a useful cell strain for virologic research. The cells grow rapidly and are maintained easily in simple fluid media on glass. The cells are readily released by trypsin from the surfaces on which they are growing, to allow the preparation of uniform suspensions of cells that can be counted and from which replicate cultures can be made. The present communication is a preliminary report of the susceptibility of the Detroit-6 strain to a selected group of viruses.

Origin of Detroit-6 cells. On the 51st day of continuous culture of bone marrow from a patient with carcinoma of the lung, a number of colonies of cells appeared from which emerged cords and sheets of cells differing from the networks of fibroblastic cells characteristic of marrow cultures of this age. The individual cells were polygonal in shape and formed colonies which reached diameters of 3 to 6 mm within several days. These colonies were removed and transplanted in Porter flasks for further propagation where they grew outward on the glass and formed sheets. The sheets of cells were trypsinized and subcultures were made in 3-ounce bottles. This sequence of events is diagrammatically shown in Fig. 1. *Subculture and maintenance of cells.* The cells were transferred and grown according to the methods described by Syverton, Scherer and Elwood(3) for the HeLa strain, but with certain modifications. The nutritive medium consisted of 40% human cord or adult serum, 2% chick embryo extract and 58% Hanks balanced salt solution (CS_{40} or HAS_{40}, EE_2, BSS_{58}). In preparing the cells for transfer, the trypsinization time was extended to 110 minutes to produce a uniform cellular suspension. An inoculum of 700,000 cells in 8 ml of medium was used to initiate growth in 3-ounce prescription bottles. The cultures were fed by removing 5 to 6 ml of the old medium and replacing it with

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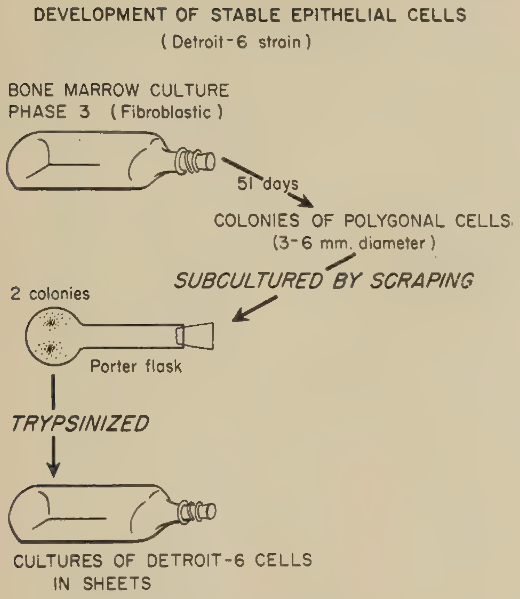

FIG. 1. Isolation of Detroit-6 strain of human epithelial-like cells.

fresh nutritive solution. Feedings were indicated when the pH was suboptimal for satisfactory propagation. The Detroit-6 cells required feedings more often than similar cultures of the HeLa strain, *i.e.* usually every 48 hours. In about 5 days the cell population increased about 5-fold. *Morphologic characteristics of the cells.* Illustrations of individual cells and sheets of the Detroit-6 strain were presented previously(1). Individual Detroit-6 cells are indistinguishable from HeLa cells, but the growth and topographic appearance of the Detroit-6 cultures present some differences from those of HeLa cultures. Following transfer of Detroit-6 cultures, individual cells and small clumps of 2 to 10 cells are dispersed on the glass. Then more aggregates appear with a reduction in the number of dispersed individual cells. These aggregates often assume the shape of cords and roundish clumps. Both individual cells and cell aggregates appear to have a degree of mobility and migrate to some extent on the glass surface, coalescing with other aggregates. Later, a solitary sheet covering the entire glass surface forms as a result of outgrowth from the individual clumps. In older cultures, the epithelial sheet has a tendency to contract, leaving vacant areas.

Virus methods. Preparation of bottle cultures for virus studies. Approximately 700,000 cells in 8 ml of nutritive medium were inoculated into 3-ounce prescription bottles. After 3 to 4 days of incubation at 36°C, the cells formed sheets composed of approximately 3×10^6 cells. At this point, the nutritive medium was removed from each bottle and cells were washed once with 8 ml of maintenance solution(3) 90% and inactivated rabbit serum 10% (MS_{90} RS_{10}). *Viruses.* The viruses employed were: 1) Poliomyelitis viruses, Type 1 (Mahoney), Type 2 (MEF_1), and Type 3 (Saukett), obtained from Dr. I. W. McLean, Jr., Parke Davis Co., Detroit; 2) Coxsackie virus, Group B, Type 1 (Conn-5), originally obtained through the courtesy of Dr. J. L. Melnick, Department of Preventive Medicine, Yale University School of Medicine; 3) herpes simplex virus recently isolated in this laboratory; 4) influenza A virus (PR8), influenza A′ virus (FM1), and influenza B virus (Lee), obtained from the Viral and Rickettsial Registry of the American Type Culture Collection. *Seed preparation of viruses.* Poliomyelitis type 1 and Coxsackie viruses were maintained in HeLa cell tissue cultures following, in general, the methods described by Syverton(3). Poliomyelitis viruses types 2 and 3 were received as supernate from monkey kidney cultures and were used in these experiments without previous passage in our laboratory. Herpes simplex virus was prepared from herpes-infected chorio-allantoic membranes of chick embryos (4). Influenza viruses were prepared from infected allantoic fluids(4). *Inoculation of cultures.* The seed suspensions of the various viruses were diluted with tissue culture maintenance medium to contain the desired num-

TABLE I. Multiplication of Poliomyelitis Virus, Type I, and Coxsackie Virus, Group B-1 in Cultures of Detroit-6 Strain of Human Epithelial-Like Cells.

Virus	Titer* inoculum	Titer* culture fluid, 24 hr	Cytopathogenic effect, 48 hr
Poliomyelitis	$10^{2.4}$	$10^{6.3}$	++++
Coxsackie	$10^{3.5}$	$10^{5.7}$	++++

* HeLa cell TCD_{50}.

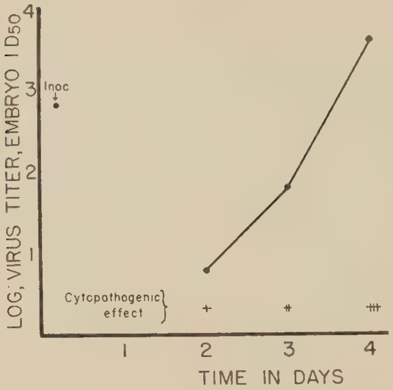

FIG. 2. Growth and cytopathogenic effect of herpes simplex virus in cultures of Detroit-6 cells.

ber of tissue culture infective doses (TCD_{50}) for poliomyelitis and Coxsackie viruses, or chick embryo ID_{50} for herpes and influenza viruses, per ml of inoculum. Each virus was tested in at least 2 cultures by placing 1 ml of the diluted virus suspension directly on the cell sheet. Within one-half hour, 9 ml of $MS_{90}RS_{10}$, or $MS_{90}RS_{20}$ (poliomyelitis types 2 and 3), were added. Following incubation of the infected cultures at 36°C, duplicate 0.25 ml aliquots of the culture fluids were removed at intervals, placed in ampules and stored at –20°C until titrated. *Virus assays.* The poliomyelitis and Coxsackie virus-infected culture fluids were diluted in 10-fold steps in MS_{100}. Of each dilution, 0.1 ml was inoculated into each of 5 tube-cultures of HeLa cells prepared according to the methods of Syverton and Scherer(2). The tubes were observed daily for viral cytopathogenicity for 5 days, at the end of which time 50%-infectivity end-points were determined by the method of Reed and Muench. The technics for titration of herpes simplex- and influenza-infected culture fluids have been previously described(4).

Results of virus infectivity tests. The data illustrated in Table I and in Figures 2 and 3 represent typical results with the various viruses. *Poliomyelitis viruses.* As shown in Table I, type 1 poliomyelitis virus multiplied rapidly to reach a titer of $10^{6.3}$ (HeLa cell TCD_{50}) in the supernatant fluid within 24 hours. This strain produced a cytopathogenic effect on Detroit-6 cells that was complete in 48 hours, *i.e.*, all cells had degenerated and had sloughed from the glass surface. Types 2 and 3 poliomyelitis viruses also produced cytopathogenic effects under the same conditions, but titrations were not carried out. *Coxsackie virus.* The effect of the Conn-5 strain was similar to that of type 1 poliomyelitis virus. The Conn-5 strain attained a titer of $10^{5.7}$ (HeLa cell TCD_{50}) in 24 hours and caused complete cellular degeneration within 48 hours. *Herpes simplex virus.* With the inoculum employed, this virus appeared to multiply less rapidly than the poliomyelitis and Coxsackie strains. As shown in Fig. 2, 48 hours after inoculation of the cells, the virus titer of tissue culture fluids was approximately $10^{0.8}$ (chick embryo ID_{50}). However this titer continued to rise through the 3rd and 4th days after infection. Concomitantly, a cytopathogenic effect first appeared in 48 hours as evidenced by small plaque-like areas of degeneration in the cellular sheet. Progressive degeneration of the cells occurred until the 4th day when most cells were affected and had sloughed. *Influenza viruses.* Under the conditions employed, influenza virus did not appear to propagate in Detroit-6 cells. This was indicated by decreases in virus titers of the culture fluids and by absence of cytopathogenicity. The activity of each influenza virus strain is illustrated in Fig. 3. Although the titers of the inocula in several experiments were higher than was desired, no PR8 virus was detected 48 hours later. The titers of the Lee and FM1 strains decreased less rapidly over a period of 4 days. A second culture in-

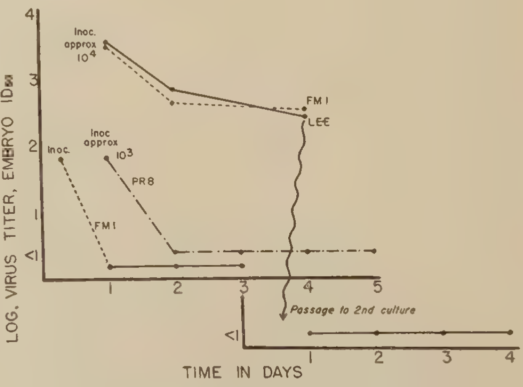

FIG. 3. Decrease in titer of influenza viruses in culture fluids of Detroit-6 cells.

oculated with the 4th day Lee-infected culture fluids did not reveal detectable virus over a period of 4 days. FM1 virus, inoculated in lower concentration, could not be found subsequently in Detroit-6 cultures.

Discussion. The initiation of new stable lines of human cells is of obvious importance in the field of virology. The HeLa strain of epithelial cells, derived from a carcinoma of the cervix by Gey(5) serves as an outstanding example of what might be accomplished with such other stable strains as might become available. HeLa cells are susceptible to many different viruses as shown by Scherer and Syverton(6-8) and have played an important role in the discovery of new viruses and virus diseases(9,10). Such cells also provide a means for elucidating the biology of animal viruses, as shown by the studies of several investigators(11-13).

Establishing stable lines of human cells involves certain difficulties. First, human tissues are not always readily available. However, there is a ready source of human tissue, bone marrow, that has not been adequately exploited. Taking biopsy specimens of human bone marrow is a comparatively routine procedure in most large hospitals. Secondly, attempts to establish stable strains of epithelial-like cells which can be adapted to rapid growth in fluid media on glass, are not simple nor always successful. Relatively few lines of human cells have thus been established (2,5,14), and the HeLa strain appears to be a singular example of such a strain that has had widespread application. The isolation of the Detroit-6 strain and its maintenance for nearly a year illustrates the possibilities that exist using marrow as a tissue source. Investigations in progress at this time show promise of leading to the establishment of several similar lines of human cells.

The cell type from which the Detroit-6 strain originated is a matter of speculation at this time, even though the marrow from which it came may have contained malignant metastatic epithelial cells. Morphologic similarities of Detroit-6 and HeLa cells have been illustrated(1). It is obvious, therefore, that studies are needed for attempting identification of cell type in the case of the Detroit-6 strain. It has not been shown that morphology or *in vitro* growth characteristics are adequate criteria for differentiation of cell types, but comparison of various cell lines with regard to their virus susceptibilities has been considered as a possible means to distinguish between cell types(4). Although the observations reported herein indicate that the several viruses used affect Detroit-6 cells in a manner which is similar to that observed with HeLa cells(2), one must take into account virus variation, genetic or otherwise, with respect to their tropisms for host cells as well as differences between *in vitro* and *in vivo* susceptibilities of the cells themselves.

Summary. The susceptibility of the Detroit-6 strain of human epithelial-like cells to a selected group of viruses has been described. Whereas influenza viruses could not be propagated and did not cause cellular degeneration, poliomyelitis viruses, a Coxsackie virus, and a strain of herpes simplex virus multiplied within these cells and produced cytopathogenic effects.

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Paper Electrophoretic Study of C^{14} Fat Emulsion Cleared from Post-Heparin Rat Plasma. (21838)

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The original observation of Hahn that heparin injection is followed by clearing of alimentary lipemia in dogs(1) has elicited considerable interest concerning the exact nature and mechanism of this clearing action. This observation has also uncovered the possibility that heparin may play a role in normal lipid transport and metabolism. Recent studies indicate that heparin is intimately involved in an enzyme system, "clearing factor," that is a blood lipase of a nature different from that of any yet characterized(2, 3,4). Other work indicates that the free fatty acid binding capacity of albumin is a link in the handling of the hydrolyzed triglyceride (5,6).

Methods. To ascertain the fate of a synthetic fat emulsion cleared by postheparin plasma the following study was undertaken. A 2.15% emulsion of uniformly labelled C^{14} soy bean oil(7) was prepared with 1% alcohol-soluble phosphatide (Glidden) in 5% dextrose using a Servall Omnimixer. Dark field examination of this emulsion showed practically all particles to be less than 1μ in size. After dilution with 5% dextrose to make a 1% emulsion, 0.1 ml aliquots were pipetted into 1 ml each of oxalated rat plasma from a fasting control rat and from a fasting rat given 10 mg/kg of heparin 10 minutes previous to sacrifice. The amount of clearing was determined turbidimetrically by measuring the optical densities of the plasma alone, of the plasma immediately after addition of the emulsion, and of the plasma plus emulsion after incubation for 1 hour at 25°C. The control showed a 30% decrease in turbidity at the end of 1 hour, while the post-heparin sample showed about 75% decrease during the same period. The samples were then diluted 1 to 6 with barbital buffer in preparation for paper electrophoresis.

Paper electrophoresis was carried out on a LKB Productur, box type apparatus handling four 40x425 mm Whatman No. 1 filter strips per run. A buffer of 0.05 M sodium barbital adjusted to pH 8.6 with glacial acetic acid was used. A series of 5 drops each containing 10 λ of the diluted plasma, were pipetted onto the middle 2 cm of each moistened strip and allowed to migrate under a constant voltage of 5 v/cm for 18 hours. Of the 4 strips per run, 2 were run with the control sample, and 2 with the post heparin sample. One strip of each sample was dyed and eluted for colorimetry by a slight modification of the bromophenol blue method for protein reported by Hardwicke(8). The remaining strip from each sample was dried at 57°C, cut into 5 mm segments which, after being mounted on stainless steel discs, were counted in a windowless gas flow counter.

The results from the control and post-heparin plasma samples are given in Fig. 1. The control sample gave a protein pattern very similar to that of the post-heparin sample. The only difference large enough to be considered significant was a slightly higher retention of protein in the region immediately beyond the origin. The distribution of C^{14} activity of the two samples, however, showed a sharp contrast. The counts above background in the region immediately past the origin were over twice as high in the control sample as those in the same region of the post-heparin strip. The activity of the control strip, however, fell to background within 30 mm beyond the origin and remained within ± 3 counts of background throughout the remainder of the protein migration area and beyond. Upon repetition of this study using higher concentrations of fat emulsion and plasma, the C^{14} localization was essentially the same but with higher counting levels and a greater proportion of the activity in the region of the origin.

As can be seen from Fig. 1 the C^{14} activity in the post-heparin sample was strongly localized in the fastest moving protein fraction which presumably is albumin. This indicates

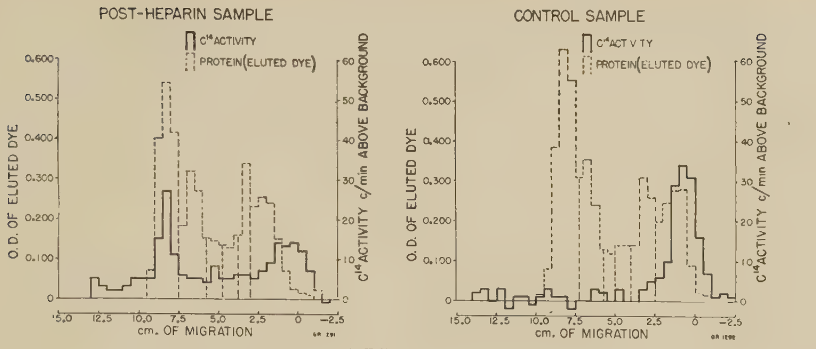

FIG. 1.

that some product of clearing is associated with this fraction. Whether the product or products of clearing are actually associated with rat plasma albumin or with some protein of similar mobility *e.g.* α1 globulin, or some normally slower moving fraction with altered mobility has not as yet been elucidated. It has also not been determined if the product of emulsion clearing localized in this fraction is free fatty acid or contains other lipid components of soybean oil as well. The presence of smaller but definite activity in regions of the electrophorogram occupied by other fractions of the cleared sample raises similar questions. These as well as other aspects of the problem are at the present time under investigation.

Summary. C^{14}-labeled fat emulsion was incubated with post-heparin plasma and the mixture was then subjected to filter paper electrophoresis. Some of the C^{14} activity was found to migrate with the albumin fraction.

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Steroid-Induced Adrenal-Pituitary Hypofunction. (21839)

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It is generally agreed that the observed adrenal atrophy following adrenal hormone treatment demonstrates decreased secretion of ACTH by the pituitary gland(1-3). Clinical evidence indicates that a hypofunctional state mirrors the anatomical depression of the adrenal cortex(4,5). Previous work reported from this laboratory(6) has shown that concurrent administration of ACTH lessens the steroid-induced adrenal depression. However, definite or direct information concerning the functional activity of the adrenal-pituitary axis has not been reported. The objective of this present investigation is to develop a standardized stress procedure which will measure secretory response of the adrenal-pituitary axis after hydrocortisone treatment. The intraperitoneal injection of potassium chloride (KCl) has been adopted as the stress-producing agent.

TABLE I. Effect of Various Doses of KCl, Injected I.P. at 5 cc/100 g Body Weight, on Intact, Adrenalectomized or Hypophysectomized Rats Fasted for 18 Hours.

No. rats	Body wt, g	Operation	Time post-operation, hr	KCl, m/l	Deaths, %
5	181	0	—	.2	100
20	174	”	—	.18	50
20	173	”	—	.16	0
10	190	”	—	.13	0
10	174	Adx	18	.16	100
9	180	”	”	.13	89
10	180	”	”	.1	90
5	170	”	”	.05	0
20	175	Hypo	”	.16	5
10	171	”	42	.16	50
5	168	”	66	.16	100
4	166	”	66	.1	0

Methods. Male albino rats of the Upjohn colony, weighing 175-200 g and fed Archer dog pellets *ad libitum,* were used throughout the experiment. All hormonal injections were given subcutaneously in 0.2 cc volume. Hydrocortisone (F) and ACTH were suspended in 16% gelatin; desoxycorticosterone acetate (DCA) was dissolved in cottonseed oil; 11β, 17α-dihydroxy-4-pregnene, 3,20-dione (21-desoxy F) was suspended in 0.5% carboxymethyl cellulose, 0.4% Tween 80, 1.5% benzyl alcohol, and .9% sodium chloride. All injections were given once daily for 10 days. Following an 18-hour fast, stress was induced by the intraperitoneal injection (I.P.) of 0.16 m KCl. In all cases the vehicle was without effect. Except where specified, adrenalectomy or hypophysectomy was performed 18 hrs prior to stress. When supportive therapy was instituted, the hormones were injected immediately after the operation and again 1 hr prior to the induction of the stress. In some instances 1% saline was substituted for the drinking water.

Results. The response of the untreated, adrenalectomized, or hypophysectomized rat subjected to various doses of KCl is listed in Table I. As can be seen, 0.16 m KCl at a dose of 5 cc/100 g body weight affects only 20% of the untreated animals. As the molarity increases, the percent deaths increases to 100% with 0.2 m KCl.

The observations recorded for the adrenalectomized or hypophysectomized rat (Table I) show that neither of these animals tolerate the stress caused by the I. P. injection of 0.16 m KCl. The former can tolerate a dosage of 0.05-0.1 m KCl; the latter is more resistant since 0.1 m solution is not fatal.

(The hypophysectomized animal differs from the adrenalectomized animal in that stress induced before 42-66 hrs postoperative does not result in death.)

Both types of animal can be protected by hormonal therapy. A dose of 1 mg DCA, 2 mg hydrocortisone, or 1% NaCl in the drinking water affords protection to adrenalectomized animals when they are stressed with 0.1 m KCl. However, this form of therapy does not give protection against 0.16 m KCl. As for the hypophysectomized animal, 5 U ACTH once daily for 3 days gives 80% protection (Table II).

The effects of 0.16 m KCl on animals injected with various hormonal preparations are listed in Table III. With increased dosages of hydrocortisone a greater mortality to the stress occurs after withdrawal of the hormone, so that at 4 mg/day/10 days 80%-90% of the animals are unable to tolerate the KCl

TABLE II. Protection Afforded by Adrenal Preparations, ACTH, or 1% NaCl against Potassium Stress to Adrenalectomized or Hypophysectomized Rats.

No. rats	Body wt, g	Operation	Time post-operation, hr	Treatment	KCl, m/l	Deaths, %
9	182	Adx	18	1 mg DCA	.16	100
9	176	”	”	” ”	.1	0
9	174	”	”	2 mg F	.16	90
10	170	”	”	” ”	.1	0
10	174	”	”	1 mg F	.1	60
9	180	”	”	1% NaCl	.16	100
10	190	”	”	” ”	.1	0
10	158	Hypo	66	5 U ACTH	.16	20

TABLE III. Effects of 0.16 m KCl on Animals Pretreated with Various Hormonal Preparations for 10 Days.

No. rats	Initial body wt, g	Treatment, 1/day/10 days	Body wt post-treatment, g	Deaths, %
40	190	0	230	10
49	196	4 mg F	164	81
20	202	3 F	177	65
6	195	2 F	207	18
26	201	6 DF	209	73
10	185	2 DCA	226	0
9	187	1 DCA	228	0
15	195	4 U ACTH	231	21

stress. (Previous work in this laboratory has shown that maximum adrenal atrophy occurred at this dosage.) Similar effects have been observed after administration of 6 mg of 21-desoxy F, in that 73% of the animals did not tolerate the stress. ACTH and DCA at the doses used had no untoward effect on the adrenal-pituitary axis as demonstrated by normal response to the stress agent.

Discussion. The response of the untreated and operated animals to the intraperitoneal injections of various dosages of KCl solution agrees with the results obtained by others (7, 8,9), in that the maximum tolerated amount was between 25-30 mg (as K^+)/100 g body weight for the normal rat and one-half or less of the dosage was fatal to adrenalectomized rats.

The experiments concerned with the adrenalectomized and hypophysectomized rats indicate that a functional adrenal-pituitary axis is essential for the animal to withstand the effects of stress. Although the adrenal hormones and ACTH at the dosage used did not raise the operated animals' resistance to normal, such therapy did partially protect the animals from an otherwise lethal dose of KCl.

TABLE IV. Effect of .16 m KCl, 5 cc/100 g Body Weight at Daily Intervals after Receiving 4 mg Hydrocortisone Daily for 10 Days.

No. rats	Initial body wt	Final body wt	Days after last inj.	Deaths, %
10	Controls	181	—	10
19	196	164	1	79
21	199	162	2	67
10	204	188	3	80
10	201	197	4	30

The degree of hypofunction of the adrenal-pituitary axis induced with hydrocortisone has been shown to be dependent upon the dosage of hormone, since increasing amounts decrease the resistance to stress. Further, rats stressed three days after the last injection of the steroid manifest a return toward normal adrenal-pituitary function (Table IV).

Animals treated with 21-desoxy F prior to the potassium stress give further indication of decreased adrenal-pituitary secretory activity. This compound has been demonstrated to inhibit pituitary secretion of ACTH in the rat without having gross glucocorticoid or thymolytic effects(10). The resultant decreased secretion of ACTH rendered the adrenal hypofunctional and, consequently, the animal was unable to tolerate the stress.

Preliminary studies indicate that the decreased resistance of the steroid-treated animals is the result of inadequate response of the pituitary gland and not adrenal hypofunction *per se*.

Summary. A standardized stress procedure utilizing potassium chloride as stressing agent has been developed to exhibit steroid-induced adrenal-pituitary axis hypofunction. Male rats treated with hydrocortisone or 21-desoxy F were unable to handle potassium chloride at a dose which was well tolerated by untreated animals.

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An Hyperglycemic-Glycogenolytic Response to Pituitary Growth Hormone in Man.*† (21840)

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Attempts to demonstrate a metabolic response to the pituitary growth factor in man have been beset with difficulties and have yielded contradictory results. In long-term balance studies some investigators have demonstrated a growth-promoting effect in subjects with pituitary insufficiency; others have not(1-6). Recently, certain acute responses to growth hormone when it was infused intravenously in man have been reported. Carballeira *et al.*(7) described transitory hyperglycemia and ketonemia and a sustained lowering of blood amino nitrogen levels and of urinary amino nitrogen excretion in subjects receiving infusions of growth hormone in amino acid solutions. Bondy(8) however, detected no effect of intravenously infused growth hormone on the blood sugar or amino nitrogen levels, but did describe an increase in blood ketone levels.

This report describes our experiences with a single lot of pituitary growth factor infused intravenously into man. A prompt hyperglycemic response was regularly demonstrated and associated with a release of glucose into the hepatic vein. Inactivation of the growth-promoting and diabetogenic activities of the pituitary preparation as measured in rats resulted in loss of the hyperglycemic response in man.

Methods. The subjects for these studies were mildly ill hospital patients, with no known metabolic or endocrine disturbance or liver disease. Splanchnic blood flow and oxygen consumption and splanchnic balances of glucose, amino nitrogen, urea and ketone bodies were estimated after an overnight fast by methods described elsewhere(9-11). Blood analyses were made at 5- or 10-minute intervals during the 20-minute control period, the 30-minute infusion and the 30-minute post-infusion period. The growth hormone preparation, PR-1, obtained from Horner & Co., Ltd., Montreal, Canada, had been prepared by the technic of Raben and Westermeyer (12) from pork pituitary glands. It was supplied as a lyophilized powder in sterile vials, each containing 50 mg.¶ According to the manufacturer it had high growth activity and contained approximately 0.01 I.U. ACTH/mg, less than 0.5 I.U. gonadotropin per mg, less than 0.5 U.S.P. unit TSH/mg, and had no oxytocic or pressor activity. However, another investigator has found some antidiuretic activity on testing it in man(13). The manufacturers indicated that they felt that their assay may have considerably overestimated its TSH content since 0.5 U.S.P. unit of TSH was the smallest dose tested for comparison. The growth hormone was prepared immediately prior to infusion by adding saline and a few drops of glacial acetic acid to the vials until the pH was approximately 3.5-4.0. The contents of 2 vials were then diluted to 200 ml with 0.9% saline. The final solution was opalescent, since it was never possible to achieve complete solution. Attempts were made to inactivate the pituitary preparation by treating the material as follows: 1. The PR-1 was prepared in vials as described above and then subjected to boiling in a water bath for 30 minutes. 2. The hormone was dissolved in 0.2 N HCl, boiled for 30 minutes in

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[‖] Fellow of American Cancer Society, 1953-54.

[¶] After this study was completed it was found that many vials contained less than 50 mg, ranging from 35-50 mg. The doses recorded in this report are, therefore, only approximate.

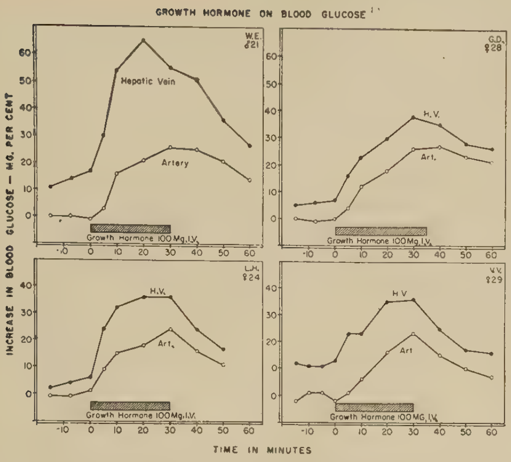

FIG. 1. Effects of intravenous infusion of 100 mg of growth hormone, PR-1, on hepatic venous and femoral arterial blood glucose levels.

a water bath, cooled and readjusted to pH 3.5 with 0.2 N NaOH. After standing for 18 hours the pH was brought to 4.5 with NaOH. 3. The hormone was dissolved in 0.1 N NaOH and immediately brought to pH 9.5 with 0.1 N HCl. 4. PR-1 was dissolved in 0.1 N NaOH, allowed to stand 18 hours at room temperature and then readjusted to pH 4.5 with 0.1 N HCl. In each case after treatment the extract was diluted to 200 ml with saline prior to infusion.

Results. Fig. 1 illustrates the influence of a 30-minute infusion of growth hormone on the femoral arterial and hepatic venous glucose levels in 4 subjects. In each case there was a prompt rise in the blood glucose levels with the maximal increase in hepatic venous glucose concentration exceeding that in femoral artery blood. On discontinuing the infusion the blood sugar values immediately began to decline toward the initial levels, which had not been reached when the experiment was discontinued after 60 minutes. The hepatic venous-femoral arterial blood glucose differences increased during the period of infusion, indicating an augmented output of glucose by the liver. This was confirmed when the net splanchnic glucose balances were calculated by multiplying the hepatic venous-femoral arterial blood glucose differences by the estimated splanchnic blood flows. These data are seen in Fig. 2, which demonstrates an unequivocal increase in glucose output in each case.

An increase in hepatic glucose output could result from an enhanced rate of gluconeogenesis from protein or from a release of glucose from liver glycogen. Measurements of splanchnic amino and urea nitrogen balances revealed no differences in these parameters during the infusion. This makes it unlikely that gluconeogenesis was responsible for the augmented glucose output and indicates that the glucose was derived from liver glycogen. Intravenous infusion of 3 mg of amorphous glucagon, which is known to produce liver glycogenolysis, results in an increase in splanchnic glucose output quite comparable to that described here(14). In view of the evidence that growth hormone stimulates glucagon secretion(16,17), it is tempting to interpret these changes as a glucagon mediated response.

There were no significant alterations in splanchnic blood flow, oxygen consumption, bromsulphthalein clearance or ketone output during these studies.

Since it is quite conceivable that the hyperglycemia during the intravenous infusion of the pituitary extract might represent a nonspecific response, such as one mediated by epinephrine discharge from the adrenal medulla, further attempts were made to relate

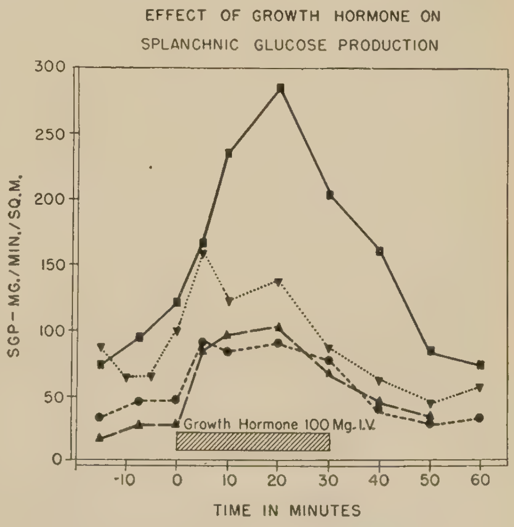

FIG. 2. Net splanchnic glucose production during intravenous infusion of growth hormone.

TABLE I. Effect of Heat and pH on Hyperglycemic Effect of PR-1 (Infusion of Approximately 100 mg over 30 Min).

	Arterial blood glucose levels during and after infusion						
				Infusion completed ↓			
Treatment	5 min.	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.
Saline acetic acid, pH 3.5							
Mean ± S.E.(6)	+4±1.7	+12±1.4	+19±1.0	+24±.6	+22±1.6	+17±2.2	+13±3
Idem, boiled		+10	+15	+14			+ 6
		+10	+19	+22	+23	+20	
	+9	+15	+18	+24	+16	+11	
Mean	+9	+12	+17	+20	+19	+16	+ 6
.2 N HCl, boiled	+2	+ 5	+16	+11	+10	+ 7	+ 5
	0	+ 3	+ 7	+ 8	+ 8	+ 5	+ 3
Mean	+1	+ 4	+12	+10	+ 9	+ 6	+ 4
Saline, pH 9.5	+5	+ 7	+14	+14	+10	+11	+ 8
	+2	+ 5	+11	+14	+13	+ 9	+ 6
Mean	+3	+ 6	+13	+14	+12	+10	+ 7
.1 N NaOH treatment	−1	+ 2	− 2	+ 1	0	+ 2	+ 5
	0	+ 3	+ 3	+ 4	− 2	0	− 1
Mean	0	+ 3	+ 1	+ 3	− 1	+ 1	+ 2
100 milliunit pitressin	−1	− 4	− 1	0	0	− 2	− 2
	−1	− 2	− 1	+ 1	− 5	− 2	+ 1
Mean	−1	− 3	− 1	0	− 3	− 2	− 1

the response more specifically to the biological properties of the pituitary extract. Glycogenolytic response to epinephrine is associated with an increase in splanchnic blood flow and oxygen consumption(15), hence it seems unlikely that the above changes are so mediated since no significant alterations in blood flow occurred during the infusions of growth hormone.

Table I records effects of heating and pH on hyperglycemic response to PR-1 infusion, as measured in femoral arterial blood. Boiling for 30 minutes at pH 3.5 did not alter the activity. When the extract was prepared at pH 9.5 or boiled in stronger acid (0.2 N HCl) there was an apparent decrease in activity, the significance of which is difficult to evaluate with only 2 observations with each preparation. There was complete lack of an hyperglycemic response in the 2 individuals who received an infusion of alkali-treated growth hormone. And finally, since Welt has found antidiuretic activity in PR-1, a control infusion containing 100 milliunits of pitressin was made, the amount of ADH estimated to be in 2 vials of PR-1. There was no hyperglycemic response to this dose of pitressin, eliminating this contaminant as the cause of the hyperglycemia.

The significance of these results is clarified in Fig. 3, details of which are given elsewhere (18,19). This figure shows the results of the assays of PR-1 for growth, diabetogenic, hyperglycemic, ketogenic and adipokinetic activities. Most significant is the fact that alkali treatment destroyed the growth-promoting and diabetogenic activities as determined in the rat as well as the hyperglycemic property in man, while all 3 activities resisted boiling at pH 3.5. At pH 9.5 there was some decrease in diabetogenic activity. Alkali treatment did not modify the adipokinetic or ketogenic responses. In contrast the acid boiling destroyed these latter activities. These differential losses of activity on chemical treatment and the correspondence between growth and diabetes in the rat and hyperglycemia in man support the interpretation that the hyperglycemia represents a true biological response to the hormone. The observations of Bornstein, Reid, and Young(16) and of Foa *et al.*(17) suggesting that growth hormone stimulates the secretion of glucagon, lead to speculation that the present effects are simi-

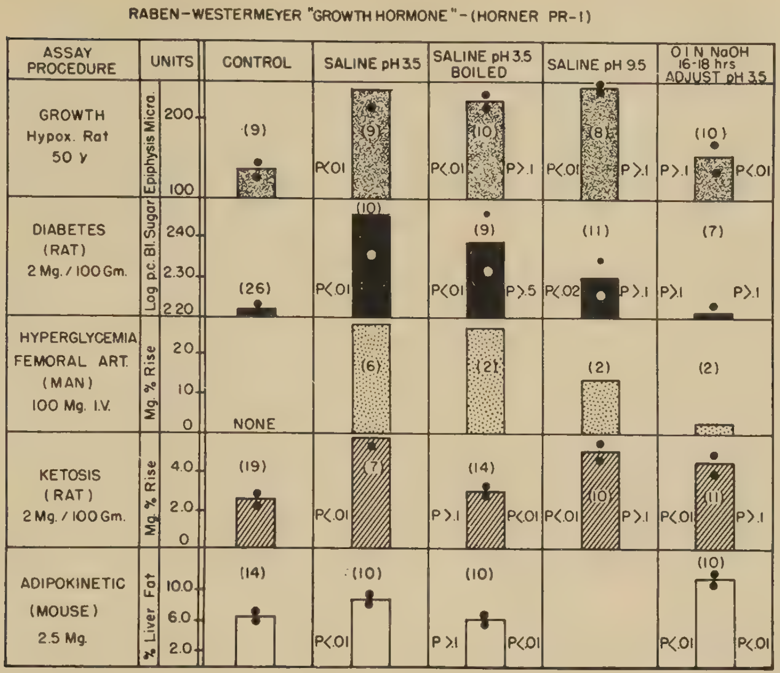

FIG. 3. Comparative metabolic activities of growth hormone. (For details of procedures see references 18,19.) "Control" column represents data of untreated animal in each test. Second column presents data for standard preparation of pituitary extract, *i.e.*, in saline acetic acid at pH 3.5. P values to the left of column refer to probability of statistical significance compared to corresponding untreated controls, those to the right compared to the standard pituitary preparation.

larly mediated, but further studies are indicated with other preparations of growth hormone.

These results were obtained with the use of a single lot of Raben-Westermeyer growth hormone. Similar transient hyperglycemia was noted by Carballeira *et al.* using a bovine growth hormone prepared by the technic of Wilhelmi, Fishman and Russell(20). Bondy (8) found no increase in blood sugar on rapid infusion but noted some elevation after prolonged infusion of both a Wilhelmi and a Raben-Westermeyer growth hormone. Our finding that the method of preparation of the hormone may influence the response might account for the different results obtained by various investigators. Until commercially available preparations of growth hormone are better standardized variable results may be obtained in different laboratories using different preparations. The future will tell which data represent responses to growth hormone *per se* and which represent artifacts or effects from contaminating pituitary factors.

Summary. 1. A rapid 30 minute intravenous infusion of 100 mg of a sample of growth hormone derived from pork pituitary glands by the technic of Raben and Westermeyer resulted in a prompt hyperglycemia in man, which lasted during the infusion and rapidly subsided thereafter. 2. The hyperglycemia was due to an augmented output of glucose by the liver and was not associated with any change in splanchnic blood flow, oxygen consumption, or amino and urea nitrogen or ketone balances. 3. The hyperglycemic response to the extract was not modified by boiling at pH 3.5, was decreased by boiling in 0.2 N HCl or when the hormone was made up at pH 9.5, and was destroyed by standing in 0.1 N NaOH for 18 hours. Growth and diabetogenic activities in the rat were likewise destroyed by alkali treatment and unmodified by boiling at pH 3.5. Ketogenic and adipokinetic activities were destroyed by acid boiling and unaffected by alkali treatment. 4.

The significance of these findings is discussed in light of the evidence that growth hormone stimulates pancreatic glucagon secretion.

We are indebted to Dr. L. Mitchell, Horner & Co., Ltd., Montreal, for generous supplies of PR-1 and to Rita Lang, Leila Holmes, Dorothy Frederick and Anna Bourne for technical assistance.

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Electrophysiological Investigation of Chlorisondamine Dimethochloride (Ecolid$^{T.M.}$) A New Ganglionic Blocking Agent. (21841)

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As early as 1914 Burn and Dale(1) described the ganglionic blocking activity of tetraethylammonium chloride. Acheson and Moe(2) and Acheson and Pereira(3) analyzed its pharmacological properties in detail. Wide clinical application of the principle of ganglionic blockade resulted from the discovery of the more specific methonium compounds (Paton(4)). Shorter acting compounds like azamethonium (Pendiomid®-Bein and Meier(5)) or trimethaphan camphor-sulfonate (Arfonad®-Randall, Peterson and Lehmann(6)) are preferred for conditions in which an acute effect is desirable. Examples of these applications are hypotensive anesthesia (*e.g.* Guiot and Damoiseaux(7), Sadove(8)) or the treatment of the acute phase of pulmonary embolism (Schneider(9), Mueller(10)). Hexamethonium (Paton(4)) and pentapyrrolidinium bitartrate (Freis *et al.*(11)) are widely used in the treatment of severe arterial hypertension, mainly because they are effective by the oral route of administration and possess a more prolonged blocking activity. Plummer *et al.*(12) described a series of isoindolines which are characterized by a particularly strong and prolonged ganglionic blocking effect. Trapold *et al.*(13) and Plummer, Trapold, Schneider, Maxwell and Earl(14) reported that one compound of this series (SU 3088, 4,5,6,7, - tetrachloro-2-dimethylaminoethyl-isoindoline dimethochloride, chlorisondamine dimethochloride, Ecolid $^{T.M.}$) had the most pronounced and pro-

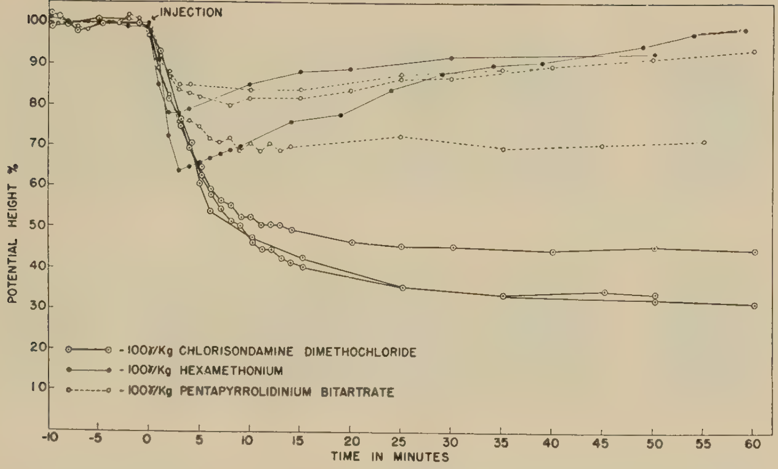

FIG. 1. Effects of ganglionic blocking agents on height of action potential recorded from the superior cervical sympathetic ganglion of Dial anesthetized cats. Maximal stimulation at a continuous rate of 1/sec. Chlorisondamine dimethochloride showed about twice the potency of hexamethonium and pentapyrrolidinium; note the lack of recovery during the period of test.

longed ganglionic blocking activity in both oral and intravenous administration in dogs. Grimson(15) subsequently confirmed the activity of this compound in man.

It is the purpose of this report to characterize the action of chlorisondamine dimethochloride by electrophysiological technics and to compare its effects to those of known ganglionic blocking agents.

Material and methods. A total of 32 cats anesthetized with Dial (60-90 mg/kg i.p.) were employed for this study. In 23 experiments the superior cervical sympathetic ganglion was exposed and the pre- and post-ganglionic portions of the cervical sympathetic trunk were exposed. The pre-ganglionic portion was sectioned and placed on platinum electrodes whereas the post-ganglionic part was cut peripherally at the base of the skull and placed on a pair of recording electrodes. The whole area was then covered with mineral oil. The ciliary ganglion was used for similar studies on parasympathetic autonomic ganglia in 9 experiments. Craniotomy was performed and the oculomotor nerve was exposed by removing part of the cerebral hemisphere. This nerve was cut and the peripheral end placed over stimulating electrodes. The ciliary nerves of the ipsilateral eye were then exposed by removing the supraorbital arch, cut peripherally and placed over a pair of recording electrodes. Both oculomotor and ciliary nerves were covered with mineral oil. Electrical stimulation of preganglionic fibers was performed by a Grass S-4 stimulator combined with a stimulus isolation unit. Single square wave impulses of 5-7 volts and 0.5-1.0 ms duration were normally employed at a 1/s rate. In some experiments different stimulation rates were used. The potentials obtained were amplified by Grass P-4 preamplifiers and fed into a Dumont 322 dual beam cathode ray oscilloscope, from which they were photographed with a Grass camera. Chlorisondamine dimethochloride, pentapyrrolidinium and hexamethonium were given intravenously at various doses dissolved in 0.9% sodium chloride solution.

Results. Using 1/s stimulations at the preganglionic portion of the cervical sympathetic trunk, 100 μg/kg of chlorisondamine dimethochloride caused a reduction of the height of the ganglionic potentials to 35-45% of their original amplitude (Fig. 1). This reduction of the size of the action potentials occurred gradually over a period of 15 minutes. Although some of the preparations were followed for 3 hours, no signs of recovery from ganglionic blockade could be observed with this drug. The peak effect of a dose of 100 μg/kg i. v. of hexamethonium occurred within 5 minutes after injection. The height of the

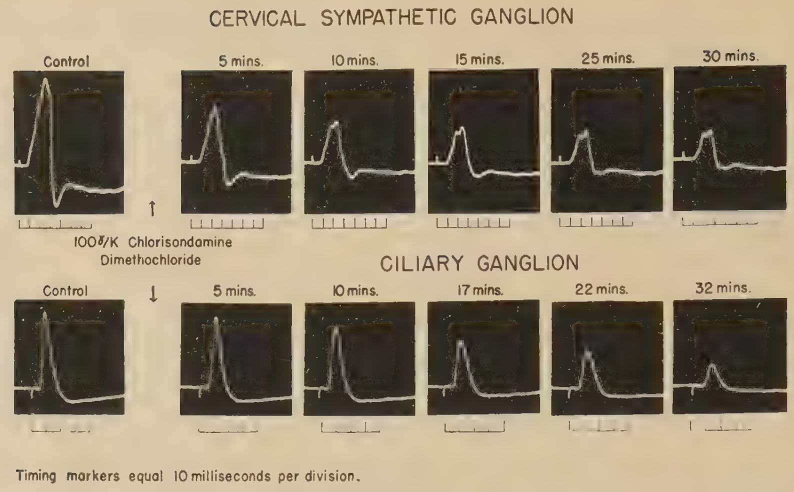

FIG. 2. Comparison of the effect of chlorisondamine dimethochloride on a sympathetic and a parasympathetic ganglion of Dial anesthetized cats. Maximal stimulation at a 1/sec. rate. Note that the potentials from both sympathetic and parasympathetic ganglia are blocked.

action potential, however, was only reduced to about 65-80% of its control values and recovery started quickly. Pentapyrrolidinium showed a peak effect of the same magnitude as hexamethonium at the same dose, but its action was more prolonged than that of hexamethonium (Fig. 1).

Since ganglionic blockade could be measured by the above preparation in the sympathetic nervous system only, the ciliary ganglion was chosen for measuring the effect of chlorisondamine dimethochloride on the parasympathetic system. As can be seen from Fig. 2 conduction through both the sympathetic and the parasympathetic ganglia was depressed by the drug. On the average the ganglionic blocking effect came on somewhat more slowly on the parasympathetic side but quantitatively the blockade fell into the same range as with the sympathetic ganglion. Recovery could not be observed in any of the experiments with chlorisondamine dimethochloride.

The rate of stimulation with single square wave impulses influenced the onset and the degree of ganglionic blocking effect of all 3 drugs. Increase in the rate of stimulation caused a more pronounced reduction in the size of the action potentials after drug application. On the other hand, slowing of the stimulation frequency resulted in a decrease of drug effect on ganglionic transmission.

Chlorisondamine dimethochloride and pentapyrrolidinium both caused an insignificant degree of blockade at a stimulation rate of 2/min., although the dose was increased to 500 μg/kg. However, hexamethonium produced about the same degree of blockade at a stimulation rate of 2/min. and a dose of 500 μg/kg as it did at a stimulation rate of 1/s and a dose of 100 μg/kg.

Discussion. The findings here described demonstrate clearly that chlorisondamine dimethochloride is a very potent ganglionic blocker of about twice the potency of pentapyrrolidinium and hexamethonium. Its duration of action is several times that of an equal dose of hexamethonium and definitely longer than that of pentapyrrolidinium.

Chlorisondamine dimethochloride has in common with pentapyrrolidinium a relatively slow onset of action which is in contrast to the more abrupt effect of hexamethonium. This is in agreement with results obtained by Plummer *et al.*(14) in the cat nictitating membrane preparation and in blood pressure experiments with anesthetized dogs. Chlorisondamine dimethochloride has a pronounced ganglionic blocking effect both on sympathetic and parasympathetic ganglia. The block appears to be somewhat more rapid on the sympathetic than on the parasympathetic ganglion but this may be due to the different location and vascularization of the ganglia involved. Winsor(16) observed rather marked blurring of the vision with the drug in patients. This may be explained on the basis of ganglionic blockade in the parasympathetic ciliary ganglion.

Why different frequencies of stimulation influenced ganglionic transmission under the effect of these drugs in different ways cannot be explained on the basis of the data so far available. From the investigations of Paton (17) it is known that certain relationships exist between the rate of stimulation applied to the superior cervical sympathetic trunk in the cat and the amount of blockade obtained with hexamethonium. This seems to be more pronounced with chlorisondamine dimethochloride and pentapyrrolidinium. Both drugs had only a very weak depressant effect on ganglionic transmission when slow rates of stimulation were used, whereas hexamethonium was still able to depress the ganglionic potential under the same conditions. This may possibly indicate a difference in the mode of action of these ganglionic blocking agents, the nature of which is as yet undetermined.

Summary. Chlorisondamine dimethochloride is a new, very potent and long lasting ganglionic blocking agent. Its ganglionic blocking potency was about twice that of pentapyrrolidinium and hexamethonium, whereas its duration of action exceeded that of hexamethonium several times. Both sympathetic and parasympathetic ganglia were blocked by the drug to a similar extent.

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Unsaturated Fatty Acids of Blood Serum from Pigs with and without Parakeratosis.[*] (21842)

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Kernkamp and Ferrin(1) have described a widespread type of dermatosis in swine and have characterized it as a parakeratosis. They suggested a nutritional or metabolic disturbance as a possible cause.

We have been studying the relation between some nutritional factors and parakeratosis, the results of which will be published in detail elsewhere. As a part of our studies we found it necessary to investigate if essential fatty acids might be involved in the disease. The rations used in our studies were not deficient in the essential fatty acids, but the possibility of a metabolic disturbance remained. Determination of the essential fatty acids in blood serum was therefore undertaken and the results of the analyses are reported in this paper.

Methods. Blood samples were drawn at 9-11 A. M., as described by Carle and Dewhirst(2). The pigs were self-fed and no restriction of feed was made before blood collections. The method of Wiese and Hansen (3) was followed for the analysis of unsaturated fatty acids. However, for one series of 6 samples (3/25/55) the purification of the fatty acids was omitted.

Results. In preliminary analyses (3/9/55) of blood from 8 healthy pigs with an average weight of 59 lb. 42 ± 2.3 mg linoleic acid, 4.0 ± 0.5 mg linolenic acid and 21 ± 1.2 mg arachidonic acid per 100 ml serum were found. In 2 new series of analyses (3/23/55 and 4/21/55) samples from both healthy pigs and pigs severely affected with parakeratosis were included.[‡] The results of these analyses are given in Table I. The content of arachidonic acid is practically the same in both groups. The level of linoleic acid is highest in blood from healthy pigs, and the difference between the 2 groups approaches significance at the 5% level of probability. Only small amounts of linolenic acid were found. It appears that there is no significant limitation in the amount of essential fatty acids in the blood serum of parakeratosis-affected pigs.

TABLE I. Average Body Weight, Age, and Content of Essential Fatty Acids in Blood Serum of Pigs with and without Parakeratosis.

	Healthy pigs	Pigs with parakeratosis
Wt of pigs, lb	86	56.6
Age " " , days	121	123
No. of samples	6	7
	mg/100 ml serum	
Linoleic acid	38 ± 4.7*	27 ± 2.1*
Linolenic acid	.7 ± .3	.7 ± .3
Arachidonic acid	23 ± 3.1	24 ± 2.4

* Stand. error of mean.

Discussion. Our analyses are relatively few, but we feel that they rule out the disturbance of essential fatty acid metabolism as a cause for development of parakeratosis. The values found for arachidonic acid are the same for healthy pigs as for pigs with parakeratosis. The content of linoleic acid is somewhat lower in blood from pigs with parakeratosis, but this may be related to the decreased feed consumption.

To our knowledge, no normal values for essential fatty acids in pig blood have been reported. Our values for pigs are somewhat lower than normal values for the human(4), which have been found to be 62-70 mg %

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[‡] The pigs affected with parakeratosis were fed either a ration composed of 84.5% ground yellow corn, 11.0% soybean oil meal, 3.0% meat scraps, 1% ground limestone and 0.5% salt or this same ration with 2% steamed bonemeal added at the expense of corn. Healthy pigs were fed the same 2 rations containing an additional 50 ppm of zinc as zinc sulfate.

for dienoic, 4-12 mg % for trienoic and 25-45 mg % for teraenoic acids.

Tucker and Salmon(5) have recently concluded that parakeratosis is due to a zinc deficiency. Our results to be published later confirm the fact that supplementary zinc is beneficial in parakeratosis. It appears therefore, that the amount of essential fatty acids in the blood of pigs is not related to parakeratosis or to zinc metabolism.

Summary. As a part of nutritional studies on parakeratosis in swine, the content of essential fatty acids in blood serum of pigs with and without parakeratosis was determined. It is concluded that low levels of the essential fatty acids in the blood are *not* associated with the development of parakeratosis.

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Effects of Cortisone, Hydrocortisone and ACTH on Mammary Growth and Pituitary Prolactin Content of Rats.* (21843)

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The role of the adrenals in mammary development and lactation is not yet clearly understood. Trentin and Turner(1) reported that adrenalectomy reduced mammary growth in rats while Smithcors and Johnston(2) found an increase in mammary growth in adrenalectomized rats. Van Heuverswyn *et al.*(3) and Nelson(4) noted that DCA stimulated duct growth in mice and guinea pigs respectively, and Mixner and Turner(5) reported that DCA had about ⅓ the mammary growth activity of progesterone in mice. Turner and Meites(6) observed that DCA did not augment the pituitary prolactin content in female rats.

Recently the mammary growth effects of ACTH and glucocorticoids have become the subject of investigation. Nelson(7) noted that crude ACTH preparations stimulated mammary growth in hypophysectomized rats. However, Flux(8) observed that cortisone, hydrocortisone and ACTH produced inhibition of mammary growth when injected into estrone-treated, ovariectomized mice of the CHI strain. When given alone, ACTH and the glucocorticoids were without effect on mammary glands of ovariectomized mice. In contrast, Selye(9) noted substantial mammary development and secretory activity when hydrocortisone and ACTH were injected into ovariectomized rats previously primed with estradiol. Selye(10) further reported that adrenalectomized and ovariectomized rats treated with hydrocortisone and estradiol showed marked mammary development and secretion. When hydrocortisone was given alone, mammary growth was reduced and was confined to the ducts.

The present study was undertaken to determine the effects of ACTH, cortisone and hydrocortisone on mammary development and pituitary prolactin content in intact female rats. Although the reports by Selye(9-10) had not yet appeared when this study was completed, our results confirm and extend his observations. In addition they show that ACTH and glucocorticoids can increase the prolactin content of the rat pituitary.

Materials and methods. Forty-two mature

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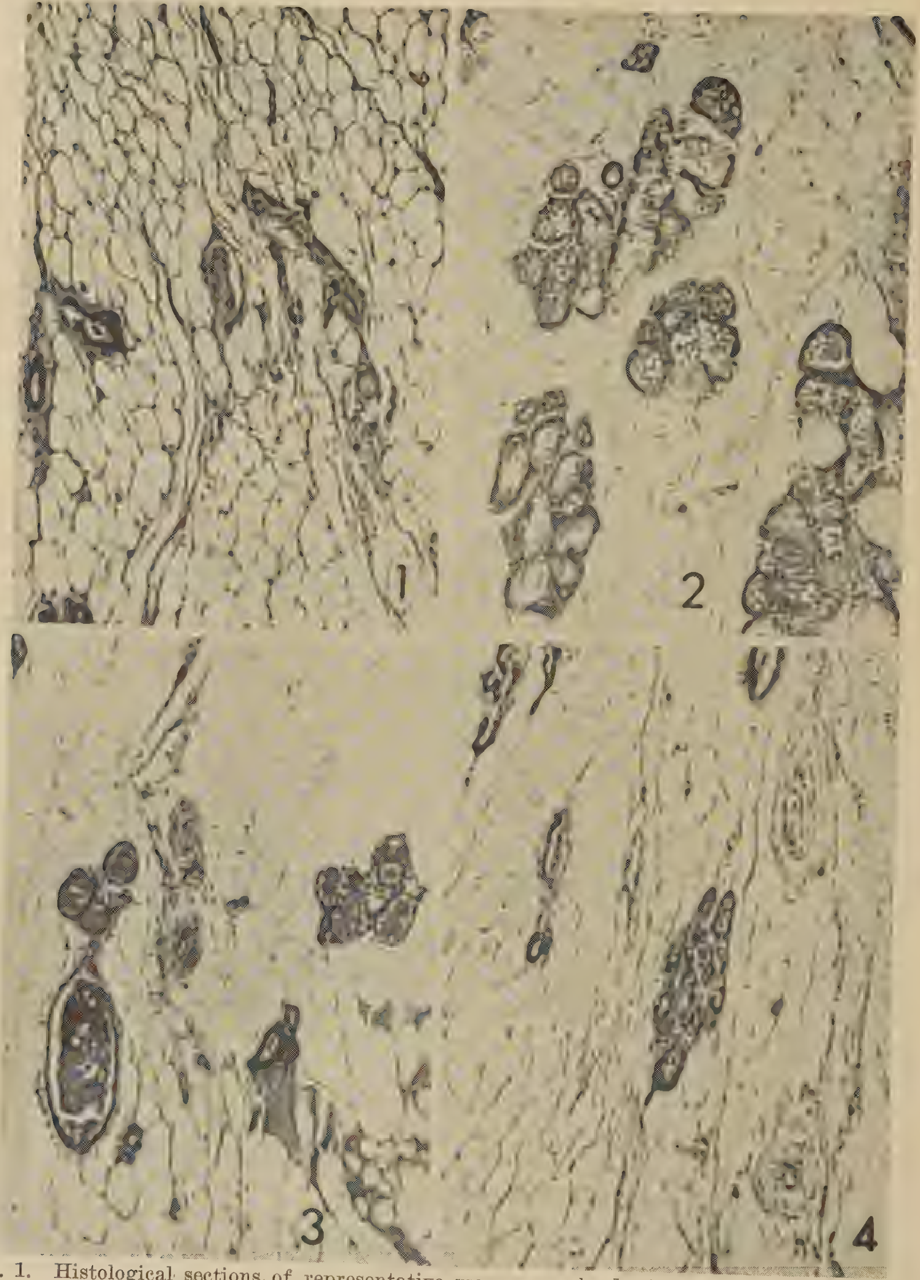

FIG. 1. Histological sections of representative mammary glands (×100) from each group: (1) 0.85% saline; (2) 2 mg cortisone; (3) 2 mg hydrocortisone; (4) 2 I.U. ACTH.

female albino rats (Carworth strain) were divided into 4 groups and injected daily for 10 days as follows: 1, controls, 0.85% saline; 2, 2 mg cortisone; 3, 2 mg hydrocortisone; 4, 2 mg (2 I. U.) ACTH (Armour's). All injections were made subcutaneously in 0.1 cc volume. On the 11th day the rats were killed and the pituitaries were removed, weighed and prepared for prolactin assay. The prolactin content of the pituitaries from each group of rats was assayed in 5 or 6 white Carneau pigeons by the sensitive intradermal method of Reece and Turner(11). In one group of 6 pigeons, the control pituitaries were injected over one crop sac and directly compared with pituitaries from hydrocortisone-treated rats, injected over the other crop sac. In another group of 5 pigeons, the pituitary suspensions from the cortisone-treated rats were directly compared with the pituitaries from ACTH-treated rats. Thus a total of 2 rat pituitaries were injected over each crop sac during a 4-day period. On the 5th day the pigeons were killed and the crop glands were removed and rated visually for degree of proliferation. The right inguinal mammary gland was dissected from each rat and prepared for gross mounting and histological examination by standard methods. Each mammary gland was rated (0 to 4) for degree of development and for secretion.

Results. The findings are summarized in Table I and Fig. 1. Cortisone and particularly hydrocortisone inhibited body growth while the dose of ACTH employed had no effect on body growth. Mammary glands of the controls showed mostly bare ducts with little branching and few to a moderate number of ductal buds. Cortisone and hydrocortisone induced marked branching of ducts and considerable lobule-alveolar development. ACTH was least effective in eliciting mammary growth, but produced moderate branching of ducts and limited lobule-aveolar growth. Cortisone was most effective in inducing secretory activity in mammary glands while hydrocortisone was only moderately effective and ACTH was least effective. There was no evidence of secretion in the control glands. Representative mammary glands are shown in Fig. 1.

Pituitary weights were not altered by any hormone treatment when compared on a body weight basis. However, all three hormones increased the prolactin content of the pituitary by all measures of comparison: Cortisone and hydrocortisone about 23 and 41% respectively, and ACTH about 71% (on a body weight basis).

Discussion. These results show that cortisone, hydrocortisone and ACTH can induce mammary growth and secretion in intact female rats. This is in accord with the findings of Selye(9-10) in ovariectomized, estrogen primed rats. In this study the greatest de-

TABLE I. Effects of Cortisone, Hydrocortisone and ACTH on Mammary Growth and Pituitary Prolactin Content of Rats.

	Avg body wt		Avg pituitary wt		Mammary gland		Avg No. pigeon units		
Treatment	Initial, g	Final, g	Actual wt, mg	Per 100 g body wt	Avg growth (0–4)	Avg secretion (0–4)	Per pit.	Per mg pit.	Per 100 g body wt
Controls (12)*	163.3	178.5 ± 8.6†	10.0 ± .6	.158 ± .012	0	0	.375 ± .06	.0375	.229
E,‡ 2 mg daily (10)*	164.8	168.0 ± 3.0	10.3 ± .9	.165 ± .01	1.6 (1-3)	3	.465 ± .11	.0451	.282
F,‡ " " (10)*	162.6	149.1 ± 4.4	10.2 ± .5	.137 ± .012	.85 (0–2.5) ± .25	2	.525 ± .21	.0316	.325
ACTH, 2 IU daily (10)*	162.3	174.6 ± 3.9	10.3 ± .6	.170 ± .003	.7 (0–2.0) ± .29	1	.640 ± .12	.0382	.394

* No. of rats per group. † Stand. error of mean. ‡ E = cortisone acetate; F = hydrocortisone acetate.

gree of mammary growth and secretion was induced by cortisone rather than by ACTH, as reported by Selye(9). The latter employed almost 25 times as much ACTH as was used in the present experiment. The lesser degree of mammary growth noted with hydrocortisone as compared to cortisone in the present experiment may be due to overdosage with the latter, as indicated by considerable loss of body weight by these rats.

Moderate increase in pituitary prolactin content produced by gluco-corticoids and ACTH suggests that these hormones may have a role in initiation and maintenance of lactation. However, they cannot be considered essential for initiation of lactation at parturition in the rat, since adrenalectomy during pregnancy does not interfere with onset of lactation or increase of pituitary prolactin content(12). The glucocorticoids and ACTH appear to possess considerably less ability to increase pituitary prolactin in rats than estrogen, although as much or more activity in this respect than testosterone or progesterone (13).

The adrenals are believed to be essential for maintaining established lactation in rats (14-15). It is not clear however, whether the adrenals are needed because of their essential role in carbohydrate, protein and mineral metabolism or because of their action on mammary growth and pituitary prolactin secretion, or both. Further work is necessary to elucidate this problem.

Summary. 1. Physiological saline (0.85%) or 2 mg doses of ACTH, cortisone or hydrocortisone were injected daily for 10 days into 42 intact, mature female rats. All 3 hormones elicited branching of ducts, lobule-alveolar development and secretory activity in mammary glands. This was most marked for cortisone and least for ACTH at the dose levels employed. 2. Cortisone increased pituitary prolactin content by about 23%, hydrocortisone by about 41% and ACTH by about 71%. 3. It is concluded that these hormones can initiate mammary development and secretion in the intact rat, but that further work is needed to establish their normal role in these processes.

We are indebted to Dr. L. Michaud of Merck & Co., Rahway, N. J., for the cortisone acetate and hydrocortisone acetate; and to Dr. Irby Bunding of Armour Labs., Chicago, Ill., for the ACTH.

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Effect of Disinfectants and of Surface Active Agents on Bacterial Protoplasts. (21844)

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It is generally assumed that disinfectants and surface active agents probably act on the cytoplasmic membrane or on the cytoplasm of bacteria. A direct approach to this problem is difficult. Almost exclusively non-bacterial proteins were used to elucidate the physical and chemical changes caused by disinfectants and surface active agents (Putnam 1). This paper attempts to demonstrate a new line of approach to this question, based on the previous observations of Tomcsik and Guex-Holzer(2). These authors showed in phase contrast microscope that the cell bodies of a bacillus, related to *B. megaterium*, assume a spherical form following the action of lysozyme on the cell-wall. The depolymerisation of the cell-wall mucopolysaccharides, caused by lysozyme, leads first to a dilatation, later to the dissolution of the cell-wall. Parallel to the successive stages of the cell-wall dilatation, the cytoplasmic membrane is separated from the cell-wall first at the polar, then successively at the lateral region, and the protoplasts assume an ovoid and finally a perfectly regular spherical form. The spherical transformation of the cell-wall free protoplasts, due to surface tension, apparently requires an intact cytoplasmic membrane and a semi-fluid cytoplasm. In heat-killed bacteria the protoplasts are fixed in rod-shape; the cell-wall dilatation and dissolution caused by lysozyme is, however, undisturbed. When the experiment on unheated bacteria is carried out in physiological NaCl solution, few of the naked protoplasts survive for 20 hr even when kept at 4°C. Weibull(3) demonstrated that the protoplasts remain morphologically intact and consume oxygen when the lysozyme acts upon *B. megaterium* in a suspension fluid consisting of 0.2 M saccharose and 0.03 M phosphate. I observed recently that a good conservation of the protoplasts can be attained by using Ringer solution instead of physiol. NaCl.

In carrying out the present work, it was assumed that the spherical transformation of the protoplasts of the lysozyme sensitive *B. megaterium* is a sign of their intactness. This phenomenon might also be regarded as a sign of life of this microorganism just as the growth in media is. This assumption led us to study the effect of disinfectants and of surface active agents on the subsequent spherical transformation of *B. megaterium* caused by lysozyme.

Materials and methods. A microorganism described previously by Tomcsik and Guex-Holzer(4), and designated as Bacillus M, was used in this work. This strain is very similar to *B. megaterium*. It was especially suitable for our studies since, under appropriate circumstances, even a 1:80 millions dilution of the crystalline lysozyme induces the spherical transformation of its protoplasts which contain but a few inclusion bodies, usually forming a smooth surface. The non-capsulated variety was grown at 28°C on Gladstone-Fildes agar for 20 hr. A thick bacterial suspension was prepared in dist. water, the opacity of which corresponded to No. 6 of Mc Farlands Barium-sulphate standard series. An equal volume of the bacterial suspension was added to geometrically increasing dist. water dilutions of the disinfectants and kept at room temperature for one hour. Five min. before the end of this period one loopful was transferred to agar medium for sterility test. The unusually high concentration of the bacterial suspension was selected to facilitate the subsequent microscopical study and agar medium was used for the sterility test instead of bouillon to allow a rough estimation of the bacterial growth. After 60 minutes contact with the disinfectant, the bacteria were centrifuged and the sediment washed once with Ringer solution. The bacteria were then resuspended in Ringer solution by restoring the original volume. The fairly thick bacterial suspension required a total concentration of approximately 1:400 of egg-white to dissolve the

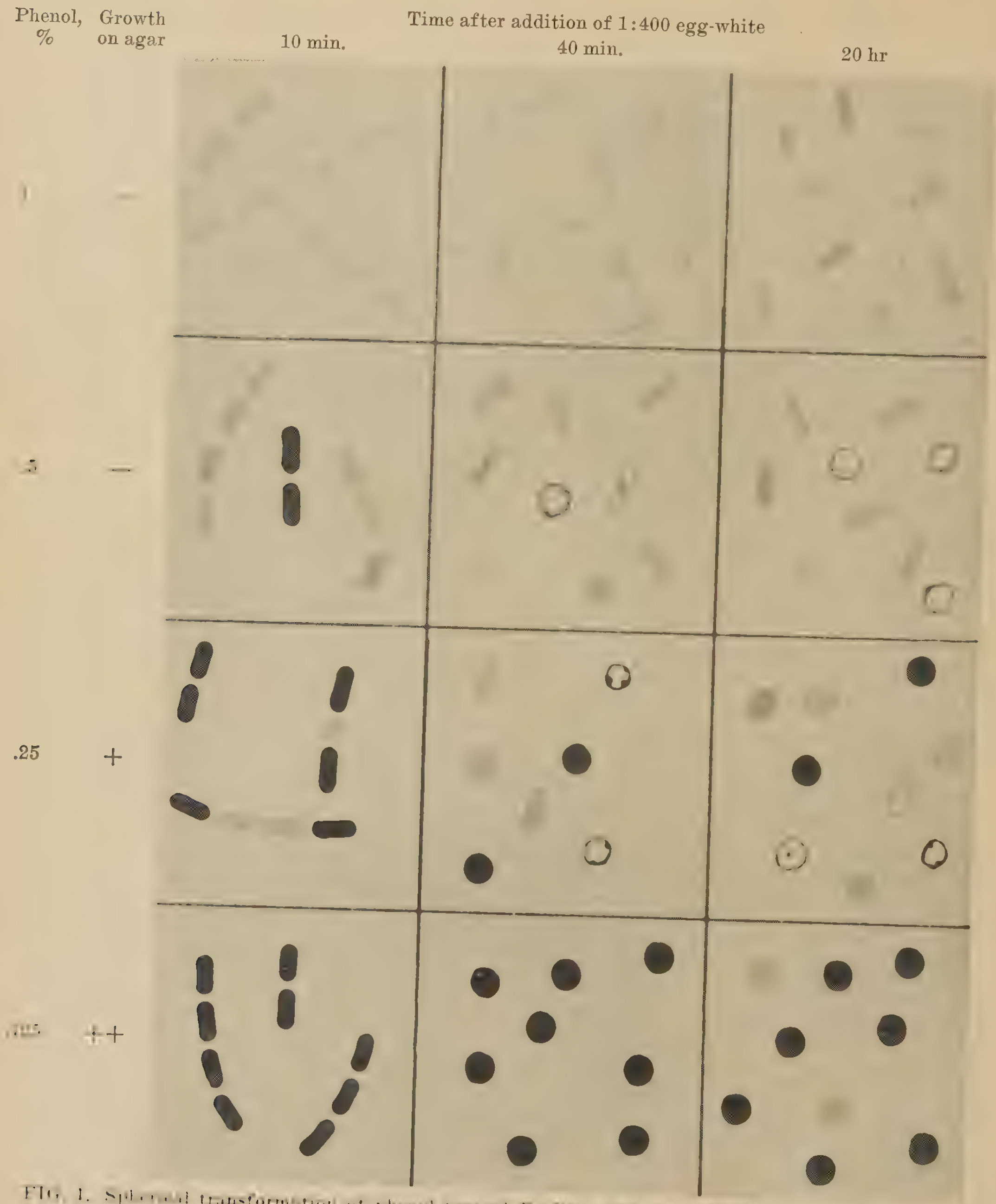

FIG. 1. Spherical transformation of phenol treated Bacillus M induced in Ringer solution by lysozyme.

cell-wall completely within 30 minutes at room temperature and to liberate from the living bacteria the naked, intact, spherical protoplasts. This concentration of egg-white was ensured by mixing 1.5 ml of the Ringer suspension of treated bacteria with 0.5 ml egg-white diluted 1:100 with Ringer solution. The readings were performed in phase contrast microscope (Wild et Co., Heerbrugg Switzerland) by using objective 85 × and ocular 10 ×. Fresh preparations were made and examined from each tube 10 and 40 minutes after the addition of egg-white. The suspensions were then kept at 4° and a third reading was performed 20 hr later. About 50% of the protoplasts obtained from living and not pretreated bacteria remained morphologically intact after this period of observation.

Results. Phenol was used to demonstrate the effect of a typical bactericidal substance on the spherical transformation of the protoplasts. This disinfectant kills the vegetative form of most bacteria after 5-10 min. exposure 1:80 to 1:110 dilution at 20°C. The non-sporulating strain of Bacillus M was killed when exposed to 1:300 phenol for 55 min. The same bacterial suspension kept for one hour in varying concentrations of phenol and then washed with Ringer solution gave the phenomenon of spherical transformation as summarized schematically in Fig. 1.
Ten min. after the addition of lysozyme the appearance of the dead bacteria in phase-contrast microscope was pale in contrast to the dark cell bodies of bacteria not killed by phenol. The chains were intact and the specific cell-wall reaction(5) when tried could be demonstrated on both pale and dark bacteria. The second microscopical examination performed 30 min. later revealed the most characteristic differences. The cell-wall was completely dissolved and correspondingly the chains were disarticulated. The cell-wall free bacteria appeared as pale rods when derived from a suspension pretreated with 1% phenol. Similar forms were obtained by Welshimer and Robinow(6) through lysozyme digestion of heat killed *B. megaterium* and designated as "small bacilli." The previous treatment with 0.5% phenol permitted occasionally the appearance of spherical protoplasts; these were, however, pale or empty. Both intact and damaged spherical protoplasts were found in a suspension treated with 0.25% phenol when the growth on agar medium was only partially suppressed. 0.125% phenol did not apparently damage the bacteria, as is shown both by the unchecked growth on agar medium and by the normal evolution of the spherical transformation of the protoplasts. These observations reveal a parallelism between the bactericidal effect of phenol and its effect in suspending the ability of the protoplasts to go over into the spherical form after the enzymatic digestion of the bacterial cell-wall. Essentially similar observations were made by killing the bacteria with potassium permanganate, Lugol solution, hydrochloric-acetic- and benzoic-acids and sodium hydroxide. The growth of the bacteria was observed on agar medium before the addition of lysozyme in a certain limiting concentration of these substances which just permitted the spherical transformation of their protoplasts after the lysozyme digestion of the cell-wall.

From the cationic surface active agents Desogen (dodecyl-methyl-phenyl-trimethyl-ammonium-metosulphate, Geigy), Bradosol (phenoxy - ethyl - dimethyl - dodecyl - ammonium - bromide, Ciba), Zephirol (alkyl-dimethyl - benzyl - ammonium - chloride, I.G.) and Sapamin (cation-active analog of $C_{17}H_{33}$–$CO{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}N(C_2H_5)_2$) were studied. The three first mentioned surface active agents are widely used in Switzerland and in Germany as disinfectants. With our standard technic, they exerted a bactericidal effect when diluted 1:24,000 *i.e.* to 1:48,000. Sapamin was very much less efficient (1:800). In lower dilutions, all of them agglutinated the bacteria which were, in spite of washing with Ringer solution, not amenable to the action of lysozyme. The chains were disarticulated by lysozyme only in 1:400 to 1:1600 dilutions and higher. Then came a zone with cell-wall free bacteria fixed in rod shape. Still higher dilutions led to marked damage and partial dissolution of the fixed bacilli, until, in certain limiting dilutions, first damaged and then intact spherical forms appeared. The normal spherical transformation of the protoplasts was observed at a dilution somewhat higher than the one just permitting growth.

From the anionic surface active agents Aerosol O.T, Mitin F. F. (Na-salt of dichlor-phenyl- (chlor/chlorsulfophenoxy/phenyl) urea, Geigy) were examined. Their action was essentially similar, regarding the spherical transformation, to that of cationic detergents with the difference that no agglutination of bacteria and no inhibition of lysozyme activity could be observed.

The disinfectants and the cationic and

TABLE I. Effect of Disinfectants and of Surface Active Agents on Lysozyme-Induced Spherical Transformation of Protoplasts.

		Before addition of lysozyme		After addition of lysozyme		
Group	Substances	Agglutination	Growth on agar*	"Small bacilli"	Damaged bacilli and spheres	Intact spherical transformation*
I	Phenol	—	300	100	200	400
	Desogen	800	48000	12000	48000	96000
	Bradosol	2400	96000	36000	72000	144000
	Aerosol O.T.	—	12000	3200	12000	24000
II	Mercuric chloride	—	100000	—	—	100

The numbers in the columns marked with (*) express the lowest dilutions of the substances giving the reaction, the others denote the highest dilutions.
— = No reaction in the dilution 1:100 or higher.

anionic surface active agents mentioned above exerted an essentially similar action on the bacterial protoplasts. In bactericidal concentrations they damaged the protoplasts and suspended their ability to spherical transformation. In contradistinction to these bactericidal agents, another group of substances could be distinguished which suppressed the growth of bacteria without apparently damaging the protoplasts. This group of substances will be exemplified here by mercuric chloride. The growth of Bacillus M was prevented on agar medium when the bacilli were exposed for 55 min. at room temperature to less than 1:100,000 dilution of mercuric chloride. The apparently "bactericidal" concentration, however, did not suspend the lysozyme-induced spherical transformation of the protoplasts after washing the bacterial suspension with Ringer solution. The lysozyme activity was somewhat retarded after treatment with 1:100 to 1:3200 dilutions of mercuric chloride, but as soon as the cell-wall dilatation was elicited, the spherical transformation could be observed even in these lower dilutions. The only difference to the normal spherical transformation consisted in the appearance of dark ovoid forms which in about 30% did not go over to the regular spheres after 20 hr. Ovoid forms occurred up to 1:6400 dilution of mercuric chloride. Essentially similar observations were made with complex mercury compounds, such as Merthiolate and Merphen. Somewhat similar results were obtained also with formaldehyde.

Some of our more essential findings, demonstrating the difference in the action of the two groups of antibacterial substances on the protoplasts, are summarized in Table I.

Discussion. The assumption that only living bacteria possessing an intact cytoplasmic membrane, and cytoplasm, are able to undergo a lysozyme-induced typical spherical transformation, was experimentally confirmed with Bacillus M treated with phenol and a number of other disinfectants, including cationic and anionic detergents. In the bactericidal concentrations of these substances, the naked protoplasts were either fixed in rod shape or revealed a disintegrated rest in rod or spherical form. The last-mentioned elements often appeared as empty spheres, indicating that the damaged cytoplasmic membrane passes into solution less easily than the cytoplasm. The fixation effect observed at higher concentrations of the disinfectants indicates a coagulation of the cytoplasmic proteins. The disintegration of the protoplasts occurring at lower concentrations points to denaturation or to the splitting of certain complexes.

It may be interesting to compare these findings with previously reported visible changes on protoplasts. Dyar(7) observed that the cytoplasm of bacteria treated with cetylpyridinium chloride appeared to have shrunk away from the cell-wall. Salton *et al.*(8) made similar observations in the electron microscope by suspending *Staphylococcus aureus*, *Streptococcus faecalis* and *Escherichia coli* in cetyltrimethyl-ammonium bromide (C.T.A.B.) Dawson *et al.*(9) studied the effect of different concentrations of C.T.A.B. on *Staphylococcus aureus* with the electron microscope. They found that by using low concentrations of C.T.A.B. autolytic disintegration intervened, causing a disintegration of the protoplasts. Intermediate concentrations cause stripping of the cell-wall, high concentrations fixation of the cell; whereby protein denaturation predominates and the autolytic enzymes are inactivated. They suggest that in some Gram-positive organisms a cytoplasmic membrane may not exist as a discrete structure. This suggestion, however, does not seem to be valid in the case of bacteria being able to form cell-wall free, spherical protoplasts. We think that the simple method described in this paper gives useful information about the action of bacterial substances on the protoplasts but has the disadvantage that its application is restricted to lysozyme sensitive bacteria.

The action of mercuric chloride and of complex mercury compounds on the protoplast is of totally different nature. These substances do not visibly damage the protoplasts and they hardly influence the lysozyme-induced spherical transformation. The apparent discrepancy between the growth inhibition of mercury compounds and the lack of their action on the protoplasts can be explained easily in view of the previous findings. Fildes(10) has shown that mercuric ions act on the protoplasts merely by combining with sulfhydryl groups and its antibacterial action is specifically neutralized by –SH compounds as glutathione and cysteine. Grumbach(11) observed that in certain microorganisms mercury compounds inhibit only the aerobic and not the anaerobic growth. Thus it must be assumed that these compounds do not elicit a denaturation of the protoplast. This assumption is proved with direct microscopic observation in the present work. Thus it follows that the ability of Bacillus M to undergo spherical transformation, following the lysozyme digestion of the cell-wall, is a more reliable sign for the life of these bacteria than the growth on ordinary agar media under aerobic circumstances. This reaction might be recommended for testing whether a substance with antibacterial activity denatures the protoplasts or not.

Summary. 1. Bactericidal agents like phenol, cationic and anionic detergents damage and denature the cytoplasmic membrane and the cytoplasm. Their direct action can be visually demonstrated on a lysozyme-sensitive Bacillus by observing the different stages in the spherical transformation of the protoplasts which occur parallel with the lysozyme digestion of the cell-wall. 2. Antibacterial agents like mercury compounds do not visibly affect the bacterial protoplast. 3. The protoplasts of killed cells are not able to undergo a spherical transformation.

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Ultraviolet Light Inactivation of Poliomyelitis Virus.[*] (21845)

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For several viruses it has been shown that inactivation by ultraviolet light proceeds exponentially and that the logarithm of the surviving fraction plotted against time gives a straight line(1). Dulbecco and Vogt(2) showed that this holds true also for poliomyelitis virus of type 1, Brunhilde strain.

In the present work the same result has been obtained with 2 strains of poliomyelitis virus, whereas one strain (MEF-1) consistently gave an inactivation curve that deviated slightly from the exponential course.

Material and methods. Virus. Poliomyelitis virus type 1: strain Mahoney, type 2: strain MEF-1 and type 3: strain Saukett, all obtained from Dr. Jonas Salk in 1952 received several passages in monkey kidney cells, thereafter 2-3 passages in HeLa cells and finally 1 passage in monkey kidney cells. The virus preparations were stored at –60°C. *Infectivity.* Infectivity measurements were performed by the plaque technic on monkey kidney plates(3,4). The titer is expressed in terms of plaque forming units (PFU) per ml. *Ultraviolet Irradiation Technic.* 25 ml of the virus preparation (tissue culture fluid centrifuged at 3000 rpm for 30 min. at 4°C) was placed in an open petri dish of 8.8 cm diameter and irradiated with a 15 watt Westinghouse "Sterilamp" at a distance of 68 cm. At this distance the lamp delivered 420 ergs/sec/cm^2. The virus suspension was stirred uniformly during the period of irradiation with a magnetic flea. 1 ml aliquots were taken initially and after irradiating for 5, 10, 15, 20, 25 and 30 minutes. The samples were stored in the dark at 4°C for periods not exceeding 24 hrs until assays could be performed.

Results. Fig. 1 shows the data obtained from one of the experiments in which each of the 3 types of poliomyelitis virus was irradiated with u.v. light. The logarithm of the surviving number of PFU per ml is plotted against the time of exposure to u.v. irradiation. It will be seen that the inactivation curves for the Mahoney and the Saukett strains are rectilinear, whereas the curve for the MEF-1 strain shows a slight but definite bend. This difference was confirmed in several experiments.

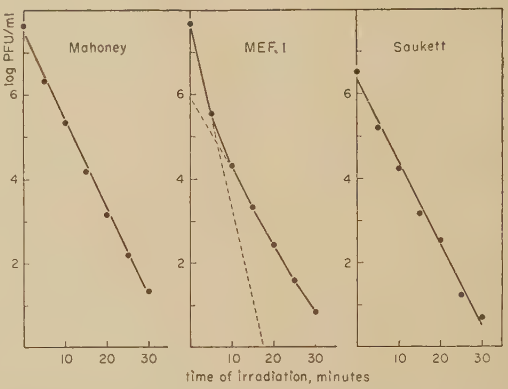

FIG. 1. Ultraviolet light inactivation curves for poliomyelitis virus type 1, strain Mahoney; type 2, strain MEF-1; and type 3, strain Saukett.

In experiments with 50-fold concentrated MEF-1 virus prepared by Schwerdt and Schaffer(5) the same slightly bending inactivation curve was found. Samples of survivors from this experiment were also assayed by intracerebral inoculation of mice, and by plotting the log ID$_{50}$ endpoints against the time of exposure to u.v. light an inactivation curve of the same shape was obtained.

Discussion. Inactivation curves that are interpreted as one-target curves often show a deviation from linearity at low survivor values. However, the difference recorded here for the 3 types of virus irradiated with u.v. light under identical conditions was so consistent and of such potential significance that it seemed worth reporting. The observed deviation could be explained if the MEF-1 virus were a "mixed population" containing a small fraction of a strain that is about twice as resistant to u.v. light as the major fraction.

Since the occurrence of various levels of resistance would be of importance in vaccine production, efforts are being made to isolate mutants of type 2 which will show no deviations from linearity on treatment with u.v. light. Needless to say such strains should be used in the production of inactive vaccines prepared by u.v. irradiation.

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It may be noted that Schwerdt and Schaffer (5) have found that purified polio virus type 2 contains about 30% ribonucleic acid. The nucleic acid fraction presumably is necessary for infectivity but not for antigenicity. It may therefore be possible to prepare a highly effective vaccine by irradiating at 260 mμ since the nucleic acid has a high specific adsorption at this wavelength and its vital structure may be preferentially destroyed without serious damage to the protein which presumably carries the antigenic property of the virus. It may be added that the nucleic acid of other viruses (tobacco mosaic virus(6), turnip yellow mosaic virus and bacteriophages(7)) is indicated to be centrally located and surrounded by protein. If this is also the case for poliomyelitis virus, inactivation by u.v. irradiation may be preferable to treatment by formaldehyde since the latter reagent may selectively affect the exterior protein groups rather than the internal nucleic acid structure.

Summary. Inactivation by u.v. light of strains of the 3 types of poliomyelitis virus (type 1: Mahoney, type 2: MEF-1 and type 3: Saukett) was found to proceed exponentially down to less than 10^{-6} survivors for types 1 and 3. For type 2 the inactivation curve consistently showed a slight bend, suggesting the presence of a small fraction of more resistant virus particles.

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Inactivation of Virus By Mustard at Varying Hydrogen Ion Concentrations.* (21846)

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Previous studies of the action of mustards upon influenza virus have shown that these chemicals may be used for selective inactivation of certain viral components and the preservation of still other viral attributes(1-3). Since cyclization of nitrogen mustards is known to be suppressed by acidic reactions and accelerated by alkaline reactions, it seemed desirable to determine the effect of mustard upon virus under varying conditions of pH. The results of such studies are presented in this paper.

Materials and methods. Preparation and treatment of virus. An egg-adapted strain of influenza A (PR8) virus was used. Virus was prepared by inoculation of embryonated eggs as previously described(1). The allantoic fluids were harvested, pooled, and centrifuged at 2500 rpm for 10 minutes; the supernatant fluid was removed and used as virus. The viral suspensions were adjusted either to pH 6.2 by the addition of 10% HCl or to pH 9.5 with 2N NaOH. After 3 hours at room temperature (25°C) the suspensions were centrifuged briefly to remove precipitated material, and the supernatant fluids were removed and treated with mustard. Control suspensions consisting of virus at pH 8.0-8.2 were handled in an identical manner.

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Mustard. The chloroethylamine derivative used in these studies was cyclohexyl [bis (β-chloroethyl)]amine hydrochloride, kindly supplied by Dr. H. B. Woodruff of Merck and Co., Inc. A solution of 0.0125 M mustard was prepared in triple-distilled water. Appropriate volumes of this solution were added to virus at room temperature to give the desired concentrations of mustard. In all cases the initial interaction of mustard and virus was allowed to proceed at 25°C, and samples were withdrawn at selected intervals for determination of infective and hemagglutinating virus. In some instances the mustard-treated viral suspensions were refrigerated an additional 18-20 hours prior to tests for virus.

Infectivity test. This was made by allantoic inoculation of 0.05 ml of serial 10-fold dilutions of virus in beef heart-infusion broth into six 10- or 11-day embryonated eggs. After 48 hours at 36°C, the individual allantoic fluids were tested for hemagglutinins by the pattern method(4) and the ID_{50} per 0.05 ml of suspension calculated by the method of Reed and Muench(5).

Hemagglutination test. The test was set up with serial 2-fold dilutions of virus and 1% chicken red cells. The test was read by the pattern method(4) after 30 minutes at room temperature. The end point was taken as the last tube showing complete agglutination and the titer expressed as the reciprocal of the dilution of virus in this tube.

Interference tests. Virus used in interference experiments consisted of (a) virus at pH 8.0 treated with 5 x 10^{-3} M cyclo mustard and (b) virus at pH 6.2 exposed to 5 x 10^{-4} M cyclo mustard. These mustard-treated suspensions were left 3 hours at room temperature and tested for residual infective virus by allantoic inoculation of 11-day embryos with 0.5 or 0.05 ml of undiluted suspension, and 0.05 ml of serial 10-fold dilutions of virus. Four eggs were used for each dilution. Mustard-treated viral suspensions which failed to yield hemagglutinating virus in these tests were used as interfering virus. Varying amounts of these interfering preparations were inoculated allantoically into groups of six 11-day embryos; immediately after this a challenge dose of active homologous virus was given. Following incubation at 36°C for 48 hr, the individual allantoic fluids were collected and titrated for their content of hemagglutinins.

TABLE I. Effect of pH upon Mustard Inactivation of Virus, 12 Samples.

(M) mustard*	pH of sample	Log ID_{50} of sample†	HA titer‡
5.0×10^{-4}	6.2	<1.0	640
2.5 "	"	<1.0	
1.25 "	"	5.0	
6.25×10^{-5}	"	6.5	
3.12 "	"	7.2	
5.0×10^{-4}	8.0	3.6	640
2.5 "	"	5.3	
1.25 "	"	7.0	
6.25×10^{-5}	"	7.7	
3.12 "	"	8.2	
0	6.2	7.7	640
0	8.0	8.2	640

* Represents final molar concentration.

† Log No. egg-infective doses left in sample after 1 hr at 25°C and 18-20 hr refrigeration.

‡ Hemagglutinin titer at time of infectivity titration.

Results. Effect of pH upon mustard inactivation of viral infectivity. The results of one of a number of similar experiments illustrating the influence of pH upon inactivation of virus by mustard are shown in Table I. It is evident that for any particular concentration of cyclo mustard, there was a greater destruction of viral infectivity at pH 6.2 as opposed to pH 8.0. The differences in numbers of surviving virus between the 2 pH values ranged from approximately one log unit for the 2 lowest concentrations of mustard to 2 to 5 log units for the 3 highest concentrations of cyclo mustard. The effect was most striking with 2.5 x 10^{-4} M mustard as evidenced by a comparison of virus samples 2 and 7. Comparison of the infective titer of untreated virus at pH 6.2 with that of control virus at pH 8.0 reveals a discrepancy of 0.5 log unit. This is within the limits of errors of the method. In any event, this difference in titer of virus between the 2 pH values would hardly account for the marked differences observed following exposure of virus to mustard.

The hemagglutinin titers of 4 of the viral samples are given in Table I. It is apparent that in this experiment there was no loss of hemagglutinating virus at pH 6.2 either be-

TABLE II. Effect pH upon Initial Stages of Destruction of Viral Infectivity by Mustard, in 12 Viral Samples.

(M) mustard × 10^{-4}	pH of sample	Min. exposure to mustard	Log ID_{50} of sample	HA titer*
5	6.2	.5	5.2	
"	"	5	<1.0	
"	"	15	<1.0	160
"	8.2	.5	5.7	
"	"	5	4.9	
"	"	15	5.0	640
"	9.5	.5	5.5	
"	"	5	5.1	
"	"	15	4.7	160
0	6.2	0	7.7	160
"	8.2	0	8.2	640
"	9.5	0	8.0	160

* Hemagglutinin titer supernate derived from centrifugation of virus following adjustment of pH.

fore or after exposure to 2.5 x 10^{-4} M mustard.

Effect of pH upon initial stages of mustard-virus interaction. Table II presents results showing the effect of pH upon the earlier stages of mustard-virus interaction. The concentration of mustard used in this and other similar experiments was 5 x 10^{-4} M. Infectivity tests carried out after admixture of virus and mustard (approximately 0.5 minute required for thorough mixing) showed an immediate drop of 2.5 log units in titer of virus. This occurred at all three pH values (6.2, 8.2 and 9.5). There was little or no further destruction of virus by mustard at alkaline reactions (8.2 and 9.5) subsequent to the marked loss during the first half minute of exposure. However, with additional exposure of virus to mustard at pH 6.2, there was a further drop in infective virus of approximately 4 to 5 log units.

The hemagglutinin titers of the control suspensions of virus are shown in Table II. In contrast to results of the experiment of Table I, there was a 4-fold loss of viral hemagglutinins at pH 6.2 in this experiment. Repeated tests showed that adjustment of pH of viral suspensions followed by centrifugation may or may not result in loss of viral hemagglutinins in the supernatant fluid, there being considerable variation in this respect with different preparations of virus. The fact that there was a loss of viral hemagglutinins at pH 6.2 failed, nevertheless, to account for the greater destruction of virus by mustard at this pH, since a similar loss of viral hemagglutinins at pH 9.5 did not result in an equivalent loss of viral infectivity.

Interfering capacity of virus inactivated by mustard at varying pH. Earlier studies(3) have shown that the interfering capacity of virus in infected allantoic fluid was destroyed by exposure of virus to 5 x 10^{-3} M cyclo mustard. Since lower concentrations of mustard are required for inactivation of viral infectivity at pH 6.2, it seemed desirable to determine the interfering capacity of viral suspensions inactivated in this manner. The results are given in Table III.

A comparison of the 2 viral preparations revealed a marked superiority of virus inactivated by 5 x 10^{-4} M cyclo mustard at pH 6.2 to cause interference. An injection of 1280 agglutinating doses of virus inactivated with 5 x 10^{-3} M mustard at pH 8.0 failed to inhibit multiplication of 316 ID_{50} of active homologous challenge virus inoculated into embryo-

TABLE III. Interfering Capacity of Virus Inactivated by Mustard at Varying pH.

AD* interfering virus	ID_{50} challenge virus	Individual HA titers	Geometric mean titers
1280 virus A	316	640, 1280, 1280, 1280, 2560, 2560	1438
640 "	"	1280, 1280, 1280, 2560, 2560, 2560	1810
128 virus B	"	<20, <20, <20, <20, <20, <20	<20
" "	3160	<20, <20, 20, 20, 40, 80	<28
" "	31600	40, 40, 80, 160, 320, 640	127
64 "	316	<20, <20, 20, 20, 80, 320	<40

* Agglutinating doses.
Virus A represents virus inactivated with 5 × 10^{-3} M cyclo mustard at pH 8.0.
Virus B " " " " 5 × 10^{-4} M " " " " 6.2.

nated eggs immediately after the interfering virus. In contrast, 128 agglutinating doses of virus inactivated with 10 times less mustard at pH 6.2 caused complete suppression of 316 ID_{50} and partial suppression of 3160 ID_{50} of challenge virus. In fact, even 64 agglutinating doses of this interfering preparation afforded marked inhibition of multiplication of 316 ID_{50} of active virus.

Discussion. A prevailing concept of mustard action assumes an activation of mustard molecules in polar solvents and intramolecular transformations leading to production of unstable and reactive cyclic intermediates. Since the formation of these cyclic intermediates is repressed by acidic reactions, the present investigations were initiated to study the modifying influence of pH upon mustard inactivation of viral infectivity.

Summary. A more complete destruction of virus occurred at acid reactions (pH 6.2) as opposed to alkaline reactions. The results with 5 x 10^{-3} M mustard also indicated that at alkaline reactions destruction of viral infectivity was completed during the first half minute of mustard-virus interaction. In contrast, at pH 6.2 the action of mustard upon virus extended beyond this half minute and led to complete inactivation of virus which survived the initial period of reaction. 2. A comparison of the interfering capacity of virus inactivated with 5 x 10^{-3} M mustard at pH 8.0 with that of virus treated with 5 x 10^{-4} M mustard at pH 6.2 revealed a marked interfering effect for the latter. This observation would appear to suggest a convenient procedure for preparation of interfering suspensions of virus for studies of the interference phenomenon.

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Relationship Between Isoniazid Resistance, Endogenous Catalase Activity and Guinea Pig Pathogenicity of *Mycobacterium tuberculosis.* (21847)

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Since the advent of isoniazid in the chemotherapy of tuberculosis, several reported studies have shown that isoniazid-resistant mutants of tubercle bacilli may not be fully pathogenic for guinea pigs(1-4). Though isoniazid-resistant tubercle bacilli may be attenuated for guinea pigs, they retain all or nearly all of their pathogenicity for CF-1 white mice.

Middlebrook(5) has shown that isoniazid-resistant mutants of tubercle bacilli recovered *in vitro* are deficient in endogenous catalase activity and that it is possible to isolate isoniazid-resistant mutants which differ in their magnitude of catalase activity(6).

Isoniazid-resistant, catalase-positive mutants are partially attenuated for guinea pigs, whereas isoniazid-resistant, catalase-negative mutants are more highly attenuated for this normally susceptible animal(7).

This communication reports the results of quantitative studies on the relationship between isoniazid resistance, endogenous catalase activity, and guinea pig pathogenicity of strains of mammalian type tubercle bacilli.

Methods. A modification of Dubos liquid medium, known as 7H3(8), was used for culturing the strains of tubercle bacilli studied.*

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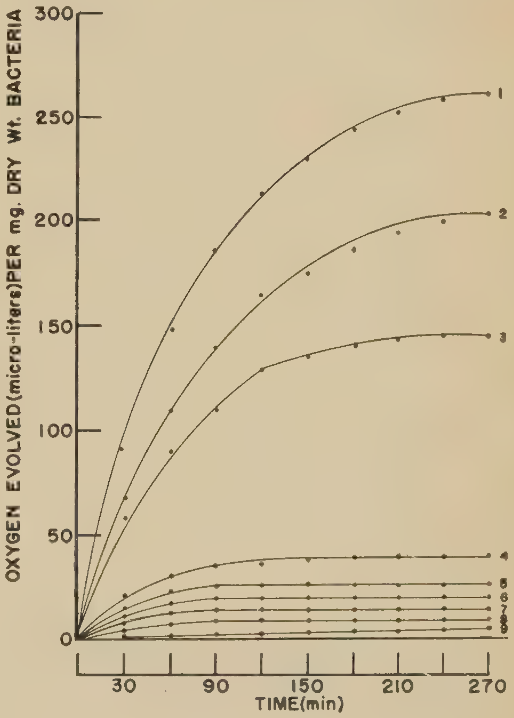
* Strains Vallee, Schierse, Clift 1A, Clift 1B, Kaminski, MK No. 1A, MK No. 10, and Kowski were furnished for study by Dr. Gardner Middlebrook, Chief, Research and Laboratories, National Jewish Hospital, Denver, Colo.

All cultures were incubated at 35°C for 18 days. Determination of the cell mass was made by optical density (OD) determinations (compared to a previously prepared curve relating OD to mg dry weight) using a Lumetron Colorimeter with a filter of 465 mu(9). Suspensions of the different strains of tubercle bacilli were adjusted to the same optical density prior to analysis. The bacterial suspensions were prepared to contain approximately 0.75 mg dry weight cells when used for comparative catalase studies. Cell suspensions showing initial slight catalase activity were concentrated by centrifugation and resuspended to one-fifth the original volume to distinguish between minor differences of activity.

To determine catalase activity, a manometric method of analysis utilizing the Warburg apparatus was used and technics of analysis were similar to those of Fugita(10). Manometric determinations were made at pH 6.6, which is within the range of 4.8 to 8.5, described as optimal for catalase activity(11). To evaluate possible differences in magnitude of catalase activity at different temperatures, determinations were made at both 0°C and 37°C. The magnitude of catalase activity has been reported to be maximum at 0°C, markedly reduced above 37°C, and to cease above 52°C(12). Preliminary determinations comparing catalase activity of washed cells versus those suspended in the primary culture medium gave identical results; therefore, the results of all studies to be reported here were done on unwashed cell suspensions. There were 0.1 ml hydrogen peroxide in the sidearm of the Warburg vessel, 1.0 ml of bacterial suspension in the reaction flask and 0.1 ml of 10% KOH in the center well. The vessels were equilibrated for 15 minutes before the hydrogen peroxide was tipped in. Final concentration of H_2O_2 was 0.1 M. Readings at 0°C were taken every 30 minutes. Readings at 37°C were taken every 10 minutes. All isoniazid susceptibility-resistance determinations were made by the Felsen plate technic(8).

The pathogenicity of strains recently isolated from patients was determined by injecting 1.0 ml of the cell suspensions of each strain (0.75 mg dry weight tubercle bacilli) subcutaneously into the right inguinal region of each of 3 tuberculin-negative 200 g guinea pigs. Surviving animals were sacrificed at 10 weeks and the gross tissue involvement was graded accordingly: A 4+ reaction indicates involvement at the site of inoculation, regional lymph nodes, liver, spleen and lungs. A 3+ reaction indicates infection in 4 of the 5 sites of involvement. A 2+ reaction indicates involvement of liver, spleen and lymph nodes and a 1+ reaction, the lymph nodes alone. A plus-minus indicates only a mild reaction at the site of inoculation.

Results. The data shown graphically in



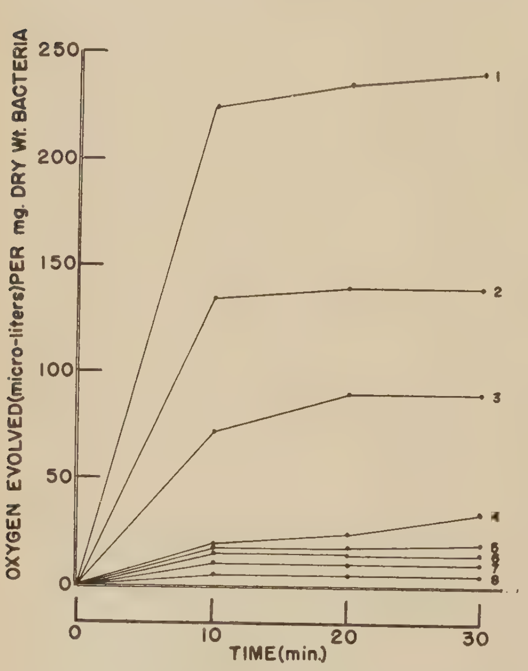
No.	Strain	Resist. to isoniazid (μg/ml)
1	H37Rv	Susceptible
2	BCG	”
3	Vallee	”
4	Schierse	5.00
5	299 L	5.00
6	Clift 1b	15.00
7	MK 1a	15.00
8	Clift 1a	15.00
9	Kaminski and MK 10	15.00

FIG. 1. Endogenous catalase activity of tubercle bacilli at 0°C and relationship to levels of isoniazid resistance.

Fig. 1 and *2* indicate an inverse relationship exists between mutants of tubercle bacilli resistant to certain levels of isoniazid and their endogenous catalase activity. As the level of resistance to isoniazid increases, there is a concurrent decrease in catalase activity. Two isoniazid resistant mutants, Kaminski and MK#10, exhibited minimal catalase activity at 0°C and none at 37°C. The isoniazid susceptible organisms, H37Rv, and Vallee (bovine) were not used in pathogenicity studies since they were not recently isolated from patients, nor was BCG (Phipps strain) used. The data in Table I show the relationship between catalase activity at 0°C, the level of isoniazid resistance, and pathogenicity for guinea pigs of typical tubercle bacilli freshly isolated from isoniazid-treated patients. Attenuation of the isoniazid-resistant mutants of tubercle bacilli for guinea pigs was found to be correlated with an increase in drug-resistance and to a decrease in catalase activity.

TABLE I. Relationship between Catalase Activity, Resistance to Isoniazid and Guinea Pig Pathogenicity.

Strain	Catalase activity (0°C), μl O_2/mg dry wt	Isoniazid resistance (μg/ml)	Guinea pig pathogenicity
Schierse	39	> 5 <15	++++
299L	23	> 5 <15	+++
Clift 1b	16	> 5 <15	++
MK 1a	13	>15 <25	+
Clift 1a	11	>15 <25	+
Kaminski	1	>25 <50	+
MK 10	1	>15 <25	±
282L	0	>25 <50	neg.
Kowski	0	>50	±



No.	Strain	Resist. to isoniazid (μg/ml)
1	H37Rv	Susceptible
2	BCG	"
3	Vallee	"
4	Schierse	5.00
5	299 L	5.00
6	Clift 1b	15.00
7	MK 1a	15.00
8	Clift 1a	15.00

FIG. 2. Endogenous catalase activity of tubercle bacilli at 37°C and relationship to levels of isoniazid resistance.

Discussion. Certain mutants of tubercle bacilli are resistant to the antibacterial activity of isoniazid. These resistant cells exhibit decreased endogenous catalase activity and, therefore, perhaps decreased ability to synthesize catalase. During repeated subculturing, in the absence of isoniazid, these mutants continued to exhibit this altered metabolic characteristic. As mutants resistant to increased isoniazid levels were selected, the magnitude of catalase activity decreased. Tubercle bacilli resistant to approximately the same concentrations of isoniazid exhibit varying magnitudes of catalase activity and this variation in catalase activity, in general, may be correlated to the pathogenicity for guinea pigs. According to the experiments described here the lower the magnitude of catalase activity, the lower the degree of pathogenicity for guinea pigs.

Summary. (1) Among typical human type tubercle bacilli and their isoniazid-resistant mutants, there is a relationship between degree of isoniazid resistance and the magnitude of endogenous catalase activity; as the level of resistance to isoniazid increases, the endogenous catalase activity decreases. (2) At a given level of isoniazid resistance, there is a decrease in pathogenicity for the guinea pig which, in general, is directly related to a decrease in catalase activity.

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Betaine and Methionine on Renal Hemorrhagic Degeneration in Rats Receiving 2-Amino-2-Methyl Propanol-1.* (21848)

DWIGHT J. MULFORD. (Introduced by Carl A. Bunde.)

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Wells(1) reported that both 2-amino-2-methylpropanol-1 and *aa*-dimethyltriethylcholine inhibited choline oxidation by liver homogenates. He showed also that there was only little methionine synthesis in the homogenates with choline and homocysteine when *aa*-dimethyltriethylcholine was present. Methionine synthesis did occur when betaine was substituted for choline. In another investigation Wells(2) reported that both 2-amino 2-methylpropanol-1 and *aa*-dimethyltriethylcholine markedly increased the severity of choline deficiency in young rats.

The present communication concerns a study of the effects of choline, betaine, casein, and methionine on the incidence of hemorrhagic kidneys in young male rats receiving a low choline diet containing 2-amino-2-methylpropanol-1.

Experimental and results. Weanling male rats (21 to 24 days of age, 40-55 g) of the Sprague-Dawley strain and born to mothers fed Purina Laboratory Chow, were placed in raised cages and fed the experimental diets *ad libitum* for 6 days. At the end of the 6-day period, the rats were sacrificed by decapitation and the kidneys examined for hemorrhages.

The basal low choline diet (TC 12) consisted of casein (General Biochemicals, Inc.) 18%, dry brewers yeast (Anheuser-Busch, Inc., Torula yeast) 3, agar 2, salt mixture (General Biochemicals, Inc., #XIV) 4, cane sugar 51.7, lard 19.9, Natola 0.1, calcium carbonate 1.0, and 1-cystine 0.3. 2-amino-2-methylpropanol-1 was added to the mixture at a level of 10 mg per gram. The effects of the addition of choline chloride, betaine hydrochloride, casein, and DL-methionine on the incidence of hemorrhagic kidneys are shown in Table I.

The animals in most of the groups not receiving choline consumed approximately 4.5 to 5.5 g of food per day before their kidneys became hemorrhagic. The animals receiving protective levels of choline consumed approximately 5.5 g of food per day. Each animal that developed damaged kidneys gained between 4 and 12 g in the 6-day period. The protected animals gained on the average between 12 and 20 g in the 6-day period. The animals receiving 129.06 x 10^{-3} mM of added DL-methionine per gram of food ate on the average only 3.7 g of food per day and gained between 1 and 7 g in the 6-day period. This decreased food consumption and growth rate

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TABLE I. Effects of Choline, Betaine, Casein, and Methionine on Incidence of Hemorrhagic Kidneys in Young Male Rats Receiving 10 mg of 2-amino-2-methylpropanol-1 per g of Diet TC 12.

Added supplement	Amt added per g of food, mM × 10^{-3}	No. of animals	Animals with hemorrhagic kidneys, %
None	—	142	98
Choline chloride	3.59	15	100
	7.17	30	27
	10.76	15	0
	14.34	30	0
Betaine hydrochloride	14.34	15	100
	50.19	30	97
	64.53	15	80
	388.50*	19	100
	78.87†	15	73
DL-Methionine	21.51	15	80
	43.02	15	86
	129.06	57	22
6% casein‡		16	100
12% ” ‡		16	100
18% ” ‡		16	100
24% ” ‡		33	100

* Each animal received 388.50 × 10^{-3} mM of betaine as a daily intraper. inj. The solution was adjusted to pH 7.0 before inj.

† Each animal received 293.97 × 10^{-3} mM of betaine by intraper. inj. daily in addition.

‡ Incorporated into Diet TC 12 at the expense of sucrose.

might explain the lower incidence of kidney lesions in this group(3). The addition of choline (7.17 x 10^{-3} mM per gram of food) to the diet containing 129.06 x 10^{-3} mM of DL-methionine changed the food intake and growth rate very little, even though none of the animals had damaged kidneys.

Griffith and Mulford(4) reported that the activity of choline on the prevention of damaged kidneys in rats was approximately 3 to 4 times that of either betaine or methionine. Table I shows that the addition of 10.76 x 10^{-3} mM of choline per gram of diet TC-12 containing 10 mg 2-amino-2-methylpropanol-1 per gram of food completely protected against hemorrhagic kidneys. Betaine, when given at approximately 6 to 14 times the choline level, and methionine, when given at 12 times, were unable to completely protect the animals against hemorrhagic kidneys. Diets as high as 42% casein were ineffective also. Wells(5) has just reported results in which he too finds betaine and DL-methionine quite ineffective.

Discussion. The data presented here suggest that 2-amino-2-methylpropanol-1 interferes with the formation of choline. Determination of whether the interference is directly related to the inhibition of choline oxidase as shown by Wells(1) will have to be left for future work. The fact that in liver homogenates betaine could methylate homocysteine in the presence of *aa*-dimethyltriethylcholine is evidence that the inhibitor may not interfere with the methyl transfer mechanism. Neither betaine nor methionine was able to prevent the incidence of kidney lesions due to 10 mg 2-amino-2-methylpropanol-1 per gram of food. One might postulate that 2-amino-2-methylpropanol-1 interferes with the methylation of a normal choline precursor. Stetten(6,7) found choline containing N^{15} in rats which had been fed N^{15} aminoethanol. Jukes, Dornbush, and Oleson (8) suggested that aminoëthanol was transformed to choline in 3-methylation steps. This was on the basis of their work with the "cholineless" mutant (No. 34486) of *Neurospora crassa.* They(9,10) were able to show that aminoethanol plus methionine or betaine did not prevent perosis in chicks. Both dimethylaminoethanol and choline prevented perosis. They concluded that the chick had no mechanism for methylating aminoethanol and that it obtained dimethylaminoethanol from its normal diet and from other natural sources for protection against perosis. duVigneaud, Chandler, Simmonds, Moyer, and Cohn (11) showed that dimethylaminoethanol was effective in preventing kidney hemorrhagic degeneration in young rats even though it methylated homocysteine only slightly. In a preliminary experiment we have found(12) that 56.16 x 10^{-3} mM of dimethylaminoethanol per gram of food almost completely protected against kidney hemorrhagic degeneration in rats receiving 10 mg of 2-amino-2-methylpropanol-1 per gram of food.

Summary. 1. The incidence of hemorrhagic kidneys in young male rats on a low choline diet is increaased by the addition of 10 mg of 2-amino-2-methylpropanol-1 per gram of food. Choline at a level of 10.76 x 10^{-3} mM per gram of food protects against renal hemorrhagic degeneration caused by 10 mg of 2-amino-2-methylpropanol-1 per gram

of food. Betaine, methionine, and casein at the levels studied were unable to completely prevent the incidence of kidney lesions caused by 10 mg of 2-amino-2-methylpropanol-1 per gram of food.

The author wishes to thank Anheuser-Busch, Inc., for the supply of Torula Yeast used in this investigation. He is also indebted to Mr. Robert Godfrey for his assistance in the experimental work.

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Chromogenic Leucyl Substrates for Aminopeptidase and Papain. (21849)

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Although a chromogenic substrate for the colorimetric demonstration of carboxypeptidase has been devised(1), this substrate is unsuitable for microscopic histo-chemistry (2). Gomori, however, has prepared certain amino acid naphthylamides which are applicable to both colorimetric and histochemical demonstration of tissue aminopeptidases (3). Of these the alanyl-β-naphthylamide exhibits the greatest hydrolysis rates and also appears to be the most satisfactory histochemical substrate. The present work deals with the synthesis and study of similar substrates whose configuration is known to be necessary for hydrolysis by leucine aminopeptidase.

The preparation of two chromogenic L-leucyl amino peptides is described. Their synthesis is an example of the usefulness of the mixed anhydride method(4,5) for preparing this type of chromogenic peptide. The L-leucyl derivatives were chosen not only because of the known specificity of leucine aminopeptidase(6), but also because of the action of papain on certain L-leucyl peptides (7). A preliminary study of the hydrolysis of these peptides and their carbobenzoxy derivatives by papain and by various rat tissue extracts, human serum, urine and saliva is also presented.

*Methods. Preparation of Substrates. Carbobenzoxy-*L*-leucyl-β-naphthylamide*—A solution of 6.64 g (0.025 mole) of carbobenzoxy-L-leucine (oil)(8) and 2.55 g (0.025 mole) of triethylamine in 25 ml of toluene was cooled to –5°C and 3.42 g (0.025 mole) of isobutyl chlorocarbonate added with shaking. A solid cake formed. After 20 minutes at this temperature a warm (40°C) solution of 3.58 g (0.025 mole) of β-naphthylamine in 100 ml of toluene was added with stirring. Carbon dioxide evolution began immediately. The reaction mixture was allowed to come to room temperature and stand overnight. The product was filtered off and washed with cold toluene and large amounts of hot water. Recrystallization from methanol-toluene gave fine white needles melting at 159°C. Weight of recrystallized material was 7.1 g (73% yield). $C_{24}H_{26}O_3N_2$; N (theory) 7.2, N (found) 7.1.

L-leucyl-β-naphthylamide hydrochloride—

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3.9 g (0.01 mole) of the carbobenzoxy compound prepared above was hydrogenated in 500 ml of methanol containing one equivalent of N HCl and 500 mg Palladium black. After 3 hours the CO_2 evolution ceased. The liquid was separated from the catalyst by filtration and taken to dryness *in vacuo*. The residue was dissolved in water, filtered and taken to dryness *in vacuo*. The residue crystallized under ether and was recrystallized from ethanol upon careful addition of ether. The yield was 2.0 g (68%) melting at 248-252°C with shrinking and darkening. $C_{16}H_{20}N_2O \cdot HCl$; theory C65.6, H 7.2, N 9.6; found C 66.0, H 7.4, N 9.5; $[\alpha]^{20°}_{D} = +81.0°$ (C = 2 in H_2O). The carbobenzoxy-L-leucyl-α-naphthylamide and L-leucyl-α-naphthylamide hydrochloride were prepared by the above procedure in satisfactory yields and with satisfactory elemental analyses. The melting points were: carbobenzoxy compound (amorphous solid) 158-60° after precipitation from hot toluene; peptide hydrochloride (fine needles) 148° after recrystallization from ethanol-ether.

The sample of DL-α-alanyl-β-naphthylamide used in this work was a generous gift from Dr. G. Gomori.

Testing of Substrates. The sources of tissue enzymes were homogenates of fresh male and female rat tissues in saline, cleared by the freezing-thawing method, human serum, urine, parotid, submaxillary, and whole saliva. The tissues and fluids were assayed for enzymatic activity with the above substrates by means of an azo-coupling method(9). Substrates were used in 0.0001 M concentration and tests were run at pH 7.0 in 0.025 M phosphate buffer at 25°C. Hydrolysis was stopped by adding molar acetate buffer pH 4.3 containing 10% Tween 20. The color developed upon addition of 0.5% Diazo B Red (Dupont) was compared with that of known standards at 550 mμ.

The leucyl- and alanylnaphthylamides as well as the carbobenzoxy-leucylnaphthylamides were tested as substrates for papain.[†] The experiments were performed at 39°C and pH 6.0 in 0.1 M citrate buffer. The enzyme was preincubated with cyanide and EDTA[‡] at pH 5 to 7 for 1 hour before reactions were begun. The assay flasks contained .01 M cyanide, 0.001 M EDTA, 0.352 mg protein nitrogen per ml and 0.05 M substrate. Course of hydrolysis was followed by means of an alcohol titration technic(10). The hydrolysis was also measured colorimetrically(9) after appropriate dilutions.

Results. Animal Tisues and Fluids. The data reported for tissues and fluids were obtained with the alanyl-β-naphthylamide and the leucyl-β-naphthylamide, except where otherwise stated. *Optimum pH*—The rate of hydrolysis was optimum in the pH range of 7.0 to 7.4. Rates decreased rapidly below pH 6.8 and slowly from pH 7.6 to 8.0. All results as to effect of substrate concentration on rate of hydrolysis, proportionality between the amount of enzyme and extent of hydrolysis, and course of hydrolysis in time, agreed with the data reported by Gomori for the glycyl-β-naphthylamide(3). *Rates of Hydrolysis by Rat Tissues.* In Table I are shown the micromoles of β-naphthylamine liberated per hour per gram of tissue from the alanyl- and leucyl-β-naphthylamides. The tissues were kidney, liver, pancreas, spleen, stomach and intestine of 12-week-old male and female white rats. The values shown are averages of two determinations from each tissue extract. Values did not differ more than 10%. *Rate of Hydrolysis by Human Fluids.* The naphthylamine released in micromoles per ml per hour by 30 human serum samples ranged from 1.0 to 1.5 for the alanyl derivative and from 0.7 to 1.0 for the leucyl derivative. The alanyl compound was hydrolyzed at a greater rate in all cases. The twelve urine samples tested showed activity ranging from 0.1 to 0.4 μM naphthylamine per ml per hour for the alanyl compound and from 0.08 to 0.2 μM per ml per hour for the leucyl compound. In all cases the alanyl compound was hydrolyzed at a faster rate. Twenty of 22 whole saliva samples, collected without artificial stimulation, showed significant activity ranging, for the alanyl compound, from 0.012 to 0.070 μM

[†] Mann Research Laboratories—2X recrystallized.

[‡] Ethylene diamine tetraacetic acid—Bersworth Chemical Co., Framingham, Mass.

TABLE I. Hydrolysis of Alanyl-β-Naphthylamide and Leucyl-β-Naphthylamide by Rat Tissue Extracts. μM of naphthylamine liberated/hr/g of tissue. (See text for details.)

	Kidney		Liver		Pancreas		Spleen		Stomach		Intestine	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Alanyl-β -naphthylamide	176.0	101.0	11.5	8.0	8.5	5.5	4.0	4.0	2.5	1.5	26.0	16.0
Leucyl-β -naphthylamide	181.0	106.0	17.0	20.0	15.0	8.0	16.0	17.0	3.5	2.5	36.0	23.5

per ml per hour and from 0.036 to 0.15 μM per ml per hour for the leucyl derivative. The leucyl compound was hydrolyzed at a higher rate than the alanyl compound by all samples tested. The 6 parotid and 2 submaxillary saliva samples tested showed no activity.

Effect of certain metals and enzyme inhibitors on rates of hydrolysis. The following metals in 0.001 M concentration showed the stated percent inhibition of hydrolysis of the alanyl and leucyl compounds by rat kidney extracts: Ca: 25, 25; Mg: 0, 0; Mn: 30, 20; Co: 15, 0; Cu: 85, 75. In 0.01 M concentration fluoride or citrate showed no inhibitory effects. At the same concentration EDTA showed 90% inhibition of hydrolysis of both compounds and cyanide showed 80% and 70% inhibition of hydrolysis of the alanyl and leucyl compounds respectively. None of the above substances showed any activating effects. Treatment of kidney extract with diisopropyl fluorophosphate (0.5 ml of 0.005 M solution per mg wet kidney tissue) for 30 minutes previous to addition of substrate caused no inhibition of hydrolysis. *Hydrolysis of other Leucyl-naphthylamide compounds.* L-leucyl-α-naphthylamide hydrochloride is hydrolyzed by rat kidney 30 times slower than L-leucyl-β-naphthylamide. No hydrolysis of the carbobenzoxy derivatives of these compounds could be demonstrated with equivalent amounts of any of the tissues or fluids used in the above experiments. *Effect of other enzymes.* No hydrolysis of the alanyl- or leucyl-β-naphthylamides was observed after treatment at pH 7.0 with 2 mg trypsin, 4 mg pepsin (at pH 2 or 7), or 1 mg carboxypeptidase. Two samples of chymotrypsin obtained from different commercial sources slowly hydrolyzed leucyl-β-naphthylamide but were without effect on the alanyl compound. One sample of chymotrypsin hydrolyzed the leucyl compound at about 4 times (0.015 μM per hour per mg chymotrypsin) the rate of the other. The rate of hydrolysis was unaffected by treatment of the chymotrypsin with diisopropyl fluorophosphate, suggesting that the hydrolysis was caused by an aminopeptidase impurity in the chymotrypsin samples.

Papain. In this preliminary work with papain no attempt was made to determine optimum conditions for hydrolysis of the substrates. The use of cysteine as an activator of papain was avoided since cysteine interferes with the colorimetric determination of naphthylamine. Under the experimental conditions employed to test the hydrolysis by papain, L-leucyl-β-naphthylamide was broken down at a decreasing rate; approximately 20% was hydrolyzed during the first hour of the reaction. L-leucyl-α-naphthylamide was hydrolyzed at 1/10th the rate of the β-naphthylamide. There was very slow hydrolysis of the alanylnaphthylamide. No detectable hydrolysis of the carbobenzoxyleucyl-naphthylamides was observed up to 24 hours incubation.

Discussion. No significant conclusions may be drawn from comparison of the hydrolysis of DL-alanyl-β-naphthylamide and L-leucyl-β-naphthylamide by tissue extracts and body fluids, especially in the light of recent work concerning the extremely different but significant rates of hydrolysis of D- and L-alanyl peptides by animal tissue enzymes(11). The rates are shown simply to demonstrate that there is a different pattern of hydrolysis of these two substrates in various tissues.

It is interesting to note that, whereas whole salivas showed significant peptidase activity, there was no activity demonstrated by any of the pure salivas (parotid or submaxillary) tested. It is also of interest that the leucylnaphthylamide was hydrolyzed at a higher rate in all of the whole saliva samples tested than was the alanyl compound.

Although carbobenzoxy-L-leucinamide has been shown to be hydrolyzed at a rate comparable to that of L-leucinamide by papain (12) no hydrolysis of the carbobenzoxy-L-leucylnaphthylamides by papain could be detected in the present work.

Summary. Two chromogenic L-leucyl aminopeptides have been prepared by a conventional method of peptide synthesis. These peptides are hydrolyzed by rat tissue extracts and human serum, urine and whole saliva as well as by pure papain. The L-leucyl-β-naphthylamide is more rapidly hydrolyzed by tissue extracts than previously suggested amino peptidase substrates, and hence gives promise of useful histo-chemical application.

We are indebted to John D. Rust for valuable technical assistance.

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Aggravating Effect of Cholesterol on Cardiovascular Changes in Choline Deficient Rats.[*] (21850)

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Diets rich in fat and severely deficient in lipotropic substances induce cardiac necrosis, coronary lipoidosis and aortic sclerosis in young male rats (100 to 130 g)(1). Female rats and older males are less sensitive to the ill effects of choline deficiency and have rarely exhibited the characteristic vascular signs. Lesions do not appear in these less sensitive animals in acute cardiovascular experiments although it is possible to produce coronary and aortic changes by prolonged deficiency of choline.

It is very well known that feeding cholesterol, either alone or in combination with other dietary procedures, can produce arterial lesions in several species (rabbit, chicken, guinea pig, hamster, dog)(2). Recently, lipomatous and atheromatous changes in vessels have been observed in rats fed a choline-supplemented diet, rich in fat and in cholesterol, for several months(3,4). It, therefore, seemed interesting to determine whether cholesterol would increase arterial damage in rats whose blood-vessels had been "primed" as it were, by acute choline deficiency.

Methods. In this study, the animals used were known to be rather resistant to the production of cardiovascular disease by choline deficiency. These were 70 female animals of approximately 125 g and 21 male rats of approximately 150 to 160 g weight. The com-

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[*] This work supported in part by grants from the Nutrition Foundation Inc. and the Canadian Life Insurance Officers' Assn.

[†] Research fellow of the Canadian Life Insurance Officers' Assn.

TABLE I. Composition of Diets.

	Diet WG-3	WG-4	WG-5	WG-6	WG-7
Casein	7	7	7	7	7
Peanut meal*	28	28	28	28	28
Soya protein†	5	5	5	5	5
Salt mixture‡	3	3	3	3	3
Sucrose-vitamin mixture‡	1	1	1	1	1
Sucrose	10.15	11	11	11	11
Starch	6	6	8	9	10
Lard	34	34	34	34	34
Cholesterol	4	4	2	1	—
Choline chloride	.85	—	—	—	—
Cinnamon	.03	.03	.03	.03	.03
α-tocopherol acetate§	.015	.015	.015	.015	.015
Cod liver oil‖	.01	.01	.01	.01	.01
Corn oil§	1	1	1	1	1

* Extracted with 50%, 75% and 95% ethanol.

† Water washed "alpha proteins" (Glidden Co.).

‡ For details see: *Canad. J. Med. Sci.*, 1953, v31, 135.

§ Fat-soluble vitamins dissolved in corn oil so that 1% of dietary corn oil will supply the desired amount of fat-soluble vitamins.

‖ Obtained from Ayerst, McKenna and Harrison Ltd., Montreal. Contains 200,000 I.U. vit. A and 50,000 I.U. vit. D per g.

position of our high fat choline-deficient diet is given in Table I. A considerable proportion of the choline-deficient rats fed cholesterol died from acute bilateral renal cortical necrosis(5) but most of the animals which did not receive supplements of cholesterol survived. These animals were not sacrificed until 10 weeks had elapsed to make sure that no late lesions were missed. Necropsy material was taken from heart muscle and aorta. Frozen sections 5 μ thick were stained for fat with Wilson's oil red O technique(6).

Results. Table II illustrates the aggravating effect of adding 4% and 2% cholesterol supplements to our high fat choline-deficient diets. The addition of 1% cholesterol to the diet raised the incidence of kidney and cardiovascular lesions only slightly, but a 2% supplement caused a very marked increase. Practically all animals which died, succumbed to hemorrhagic kidney lesions (HKL). There seems to be a close connection between the occurrence of HKL and cardiovascular disease. The animals on the choline-deficient cholesterol-free diet showed cardiovascular changes to a lesser degree than the ones supplemented with cholesterol. The animals receiving cholesterol (4%) plus 0.85% choline, thrived and grew well. This indicates that cholesterol, in these acute experiments, is much less harmful when choline is present in the diet.

The food consumption appeared to be higher in the cholesterol supplemented animals (female) than in the controls(7). Therefore, in the second set of experiments when 150 g male rats were used, we introduced strict pair-feeding but still found the same aggravating effect as with *ad libitum* feeding. The food consumption was calculated for only the first 7 days when all animals were gaining weight. Thereafter, food consumption became irregular due to the ill effects of choline deficiency and comparisons were impossible. All livers of choline-deficient animals were very fatty and kidney changes ranging from acute bilateral cortical necrosis to renal "frosting"(8) were very frequently observed in the choline-deficient cholesterol supplemented groups. These kidney lesions were rarely seen in the choline-deficient animals without added cholesterol. The lesions in the ehart and vessels have been described(1). The choline supplemented animals receiving 4% of cholesterol in the diet were perfectly healthy but the fat content of their livers was somewhat increased. No significant changes could be discerned in their cardiovascular systems.

Discussion. Cholesterol supplements aggravate the occurrence of renal cortical necrosis and of cardiovascular lesions in rats on a high

TABLE II. Aggravating Effect of Dietary Cholesterol on Renal and Cardiovascular Lesions in Choline Deficiency.

No. of rats	Sex	Wt, g	Dietary supplement		Food consumption (avg g/day), g	Death from HKL, %	Cardiovascular pathology			Aortic lesions in exp. animals, %
			Choline chloride, %	Cholesterol, %			Heart	Coronaries	Aorta	
10	♀	120	.85	4	14.2	0	0	0	0	0
10	♀	”	—	4	12.2	60	10	4	6	60
20	♀	”	—	2	11.5	65	12	6	8	40
10	♀	”	—	1	11.0	20	5	1	3	30
20	♀	”	—	—	10.0	25	8	4	4	20
9	♂	155	—	2	14.0*	66	6	4	5	55
6	♂	”	—	1	14.7*	33	2	1	2	33
7	♂	”	—	—	14.6	28	3	0	1	15

* These two groups were pair-fed with the last group.

fat choline-deficient diet. Cholesterol supplements of 4% and 2% are very effective and some change is seen with 1%. The concomitant increase in kidney as well as cardiovascular lesions favors the hypothesis that the cardiovascular lesions induced by acute, severe choline deficiency are aetiologically linked to kidney damage as most animals with cardiovascular lesions died either from HKL or showed at least some degree of renal "frosting." A diseased kidney might conceivably be unable to regulate phosphorus and calcium levels in plasma and tissues and the resulting electrolyte disturbance might favor the production of vascular damage. Comparable clinical conditions have recently been discussed by Fanconi(9).

Other workers have shown a relation between kidney damage and cardiovascular lesions(10-14) but the possibility that choline deficiency is primarily responsible for the production of our cardiovascular lesions cannot yet be ruled out. These cardiovascular lesions occur with lowered cholesterol levels(15) and decreased low density triglyceride-bearing lipoproteins(16) in the blood. The aetiology of the cardiovascular lesions in acute choline deficiency is different from those observed by Mann in monkeys(17), Page and Brown(3), and Wissler(4) in rats. These workers produced cardiovascular lesions in their animals by feeding cholesterol plus choline in combination with other atherogenic factors. Choline under their experimental circumstances seems to be necessary to secure an adequate food intake and to maintain the cholesterol plasma level(15), which according to these authors appears to play a significant part in the aetiology and pathogenesis of their cardiovascular lesions.

The aortic lesions observed by these authors(3,4,17) consist of intimal lipoidosis with subintimal hyperplasia while under our conditions, the changes in the aorta are mainly in the media *i.e.* degeneration, necrosis and calcification. Calcium deposits have so far not been observed in the lesions of the above-mentioned authors, while we do not find any appreciable amounts of cholesterol which is readily demonstrable in their lesions. In short, these lesions(3,4,17) are, in part, atheroma-like while ours may be compared with the Moenckeberg type of arterial disease. Although the cardiovascular lesions in choline deficiency have, as stated above, a completely different pathology, pathogenesis, aetiology and blood biochemistry as compared with the changes described by the authors, it is still possible to demonstrate the potent aggravating effect of cholesterol supplements. This suggests that cholesterol is a potent atherogenic and sclerogenic substance when the cardiovascular system is "primed" by one of the many experimental procedures which damage vessel walls.

Recent work by Pilgeram and Greenberg (18) may eventually lead to an explanation of the aggravating effect of cholesterol on choline-deficient tissues. These workers suggest that phosphatidyl-choline is necessary for normal cholesterol metabolism and that the difference in species susceptibility to cholesterol-induced atherosclerosis may be correlated with differences in their capacity to make phosphatidylcholine. Rats have a greater ability to form phosphatidylcholine than chicks and guinea pigs. These findings might help to explain why on one hand rats are rather resistant to cholesterol-induced atherosclerosis and why, on the other hand, cholesterol, by increasing the demand for phosphatidylcholine, enhances the severity of the lack of choline in a choline-deficient animal.

Summary. 1. Cholesterol supplements (4%, 2% and 1% in decreasing order of effect), increase the rate of occurrence of cardiovascular lesions in choline-deficient female and male rats which are resistant to the effects of choline-deficiency alone. 2. The significance of kidney damage in the production of these cardiovascular lesions is discussed. 3. The aggravating effect of cholesterol on choline-deficient tissues occurs in the presence of decreased serum cholesterol levels. 4. The significance of choline in the production of experimental arteriosclerosis depends upon the procedures used. Under certain circumstances the presence of choline in the diet is apparently necessary for the production of (atheroma-like) lesions. In others choline deficiency results in or favors the production of cardiovascular lesions (Moenckeberg type). The nature and the mechanism of production of the two types of cardiovascular lesions are quite different.

The authors appreciate Dr. W. S. Hartroft's initiative and advice during the course of this experiment.

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A Modified Krieg Stereotaxic Instrument for Producing Intracranial Lesions in the Rat.* (21851)

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(Introduced by R. W. Bates.)
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The stereotaxic instrument designed by Krieg(1) is considerably more convenient to use with small animals than is the standard Horsley-Clarke apparatus. Experience in the employment of the Krieg instrument indicated that certain modifications might facilitate its use. This report describes changes in the original design which have been found of considerable advantage over a trial period of 4 years.

Front and rear views of the instrument are shown in Fig. 1 and 2. The machine is simply constructed of brass and lucite. Three dimensional localization of the electrode is provided by 1½ calibrated mechanical microscope stages. To provide accurate horizontal placement of the rat's head, care must be taken to construct the center of the ear plugs 1¼ inches above the base plate and the top of the bar for the upper incisors 1-1/16 inches above the base plate. The ear bar posts are 2½ inches apart and the top of the brass plate supporting the electrode carrier is 2¾ inches above base plate.

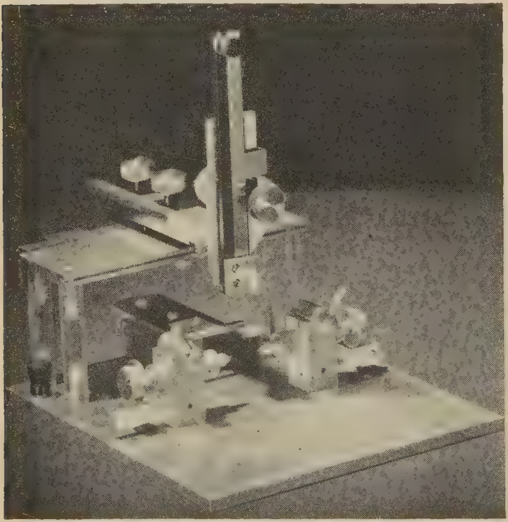

FIG. 1. Front view of stereotaxic instrument.

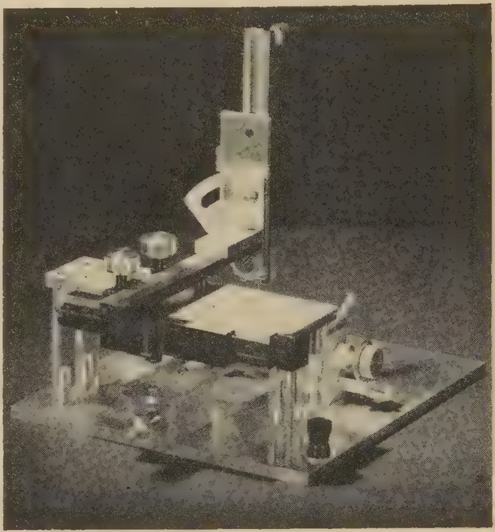

FIG. 2. Rear view of stereotaxic instrument.

The modifications of greatest value are listed below: 1) A sliding support and groove (L) which helps carry the weight of the electrode holder and greatly increases the rigidity of the mechanism. 2) Ear bars with both coarse and fine adjustment, permitting rapid insertion and removal of the animal's head. Coarse adjustment is attained by loosening the locking lever (D) permitting the entire ear bar cylinder to slide freely. When it is in approximately correct position, the locking lever is tightened and fine adjustment is attained by the screw mechanism within the cylinder. 3) An adjustable bar or "bite" (A) over which the rat's upper incisors fit. The animal's head is held down firmly with a piece of flexible rubber tubing or rubber band which can be quickly attached to the projecting ends of the cross bar. 4) Slide locks (N) for each of the 3 dimensional movements of the electrode carrier. (Not illustrated in the photographs.) These produce a friction lock

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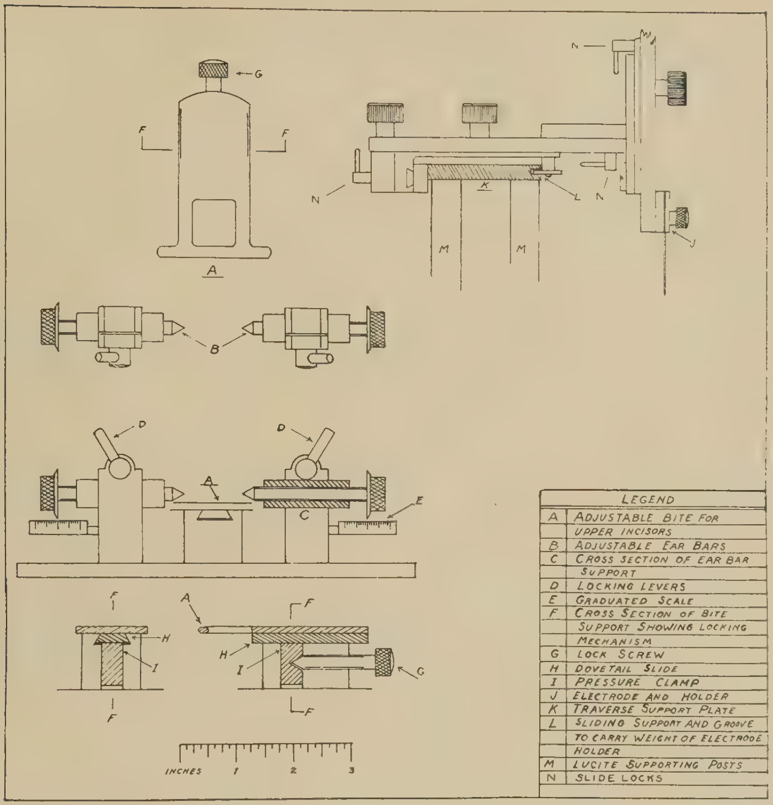
* We are indebted to Dr. E. B. Astwood for valuable suggestions on construction details.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INCHES 1 2 3
LEGEND
A ADJUSTABLE BITE FOR UPPER INCISORS
B ADJUSTABLE EAR BARS
C CROSS SECTION OF EAR BAR SUPPORT
D LOCKING LEVERS
E GRADUATED SCALE
F CROSS SECTION OF BITE SUPPORT SHOWING LOCKING MECHANISM
G LOCK SCREW
H DOVETAIL SLIDE
I PRESSURE CLAMP
J ELECTRODE AND HOLDER
K TRAVERSE SUPPORT PLATE
L SLIDING SUPPORT AND GROOVE TO CARRY WEIGHT OF ELECTRODE HOLDER
M LUCITE SUPPORTING POSTS
N SLIDE LOCKS

FIG. 3. Detail drawing of various parts.

of each of the 3 movements and prevent accidental change of position of the electrode due to vibration or jarring of the apparatus. They also permit setting the device for producing a lesion in a given area in a series of rats of equal size without the necessity of completely resetting the machine with each animal.

Using this modified instrument, it is possible to make bilateral intracranial lesions in 6-8 rats per hour. Lesions can be placed as calculated in approximately 90% of the operated animals. Its operation is the same as described by Krieg for his original instrument. Because of some differences in the dimensions of the machine from that of Krieg, it is necessary to reorient the coordinates of his atlas (1).

A further point of interest is that steel sewing or surgical needles insulated by dipping in diluted lucite cement have been found much easier to prepare and use for electrodes than the usual enameled wire.

Summary. Certain modifications in the design of the Krieg stereotaxic instrument are described which have been found to facilitate its use in the production of intracranial lesions in the rat.

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Effect of Environmental Temperature and Dietary Protein on Urinary Nitrogen Excretion of Rats. (21852)

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In recent years considerable attention has been paid to changes in metabolism during exposure to extremes of temperature, since the process of adaptation must in some manner be reflected in the biochemistry of the body. Of particular importance is nitrogen metabolism, for nitrogen is concerned in both tissue maintenance and body growth.

Most of the investigations have used the direct approach of blood and urine analysis for total nitrogen and for various nitrogenous compounds. Adolph(1) has reported that high concentrations of urea appeared in the urine of men exposed to the severe heat of the desert. Bader *et al.*(2) state that there is no significant change in the urinary uric acid/creatinine ratio or the basal metabolism of men exposed to heat or cold. Lathe and Peters(3) indicate that a low environmental temperature increases the rate of protein catabolism. Treichler and Mitchell(4) have shown that animals maintained on a low protein diet and exposed to a low ambient temperature excrete large amounts of creatinine; Selye(5) and Samaras(6) have shown an increased creatinuria in rats exposed to low temperatures. More recently, You *et al.*(7) have found that the increased nitrogen excretion of rats exposed to a low temperature was not dependent on the thyroid or adrenal gland.

It is evident from the foregoing that we have but a partial picture of shifts in nitrogen metabolism occurring during and as a result of exposure to heat or cold. Since most investigators have confined themselves to the first phases of a sudden and severe exposure to extremes of temperature, it is possible that the changes in nitrogen excretion do not persist in the animal well adapted to the experimental temperatures.

This report deals with the urinary nitrogen excretion of rats subjected to prolonged exposures to 3 experimental temperatures (4°C, 23°C, and 35°C). The temperatures 4°C and 35°C have been found to be within the physiological range of adaptation of laboratory rats. Since 23°C approaches the comfort zone of small mammals, the rats exposed to this temperature have been designated controls. Following the lead of Treichler and Mitchell(4) all rats were maintained on a low protein diet, *i.e.* a level of 5.3% protein, which appears to be adequate for normal growth and function in the rat. By using such relatively low protein diet we can more readily observe changes in metabolic potential of animals subjected to temperature stresses.

Methods and procedure. Adult (220-250 g) male Long-Evans rats (8 animals per group) were continuously exposed to the 3 environmental temperatures tested (4°C ± 0.2, 23°C ± 1.0, 35°C ± 0.8) for a period of 50 days. The animals were placed in pairs in stainless steel cages. Measurements of food intake, urinary nitrogen excretion, and body weight were made every third day from the 10th to the 50th day of exposure. For this purpose the rats were placed in pairs in metabolic cages and the urine collected from each cage under toluene for a period of 24 hours. Total urinary nitrogen was determined by micro-Kjeldahl, urea was determined by the method of Ormsby(8), ammonia and amino acid nitrogen by the formol titration method, allantoin by the method of Larson(9), creatinine by the method of Greenwald and Gross(10), creatine by the method of Folin(11), and uric acid by the method of Folin(12). All urinary nitrogenous constituents were converted to nitrogen equivalents and expressed as mg N per 100 g of rat per day. These values were then added and the sum compared with the values obtained by the micro-Kjeldahl determinations of total urinary nitrogen. In all cases good agreement was found between the total urinary nitrogen as determined by micro-Kjeldahl and that summated from the various nitrogenous fractions. Furthermore, a very close

TABLE I. Effect of 50 Days Exposure to High and Low Ambient Temperatures on Growth, Nitrogen Intake, and Urinary Nitrogen Excretion of Adult Rats. Values of nitrogen intake and urinary nitrogen are expressed as mg N per 100 g of rat per 24 hr.

Exposure temp.	35°C				23°C (control)		4°C			
		S.E.*	t*	p*		S.E.		S.E.	t	p
% change in body wt	+22.1				+29.1		+13.0			
Mean N intake	49.2	2.67	3.04	<.01	63.9	4.04	106.0	3.38	8.09	<.01
" urea N	35.1	2.69	4.07	<.01	48.6	1.95	95.4	3.67	11.20	<.01
" ammonia and amino acid N	5.79	.57	2.46	<.05	4.01	.43	4.00	.54	.01	>.90
" allantoin N	4.40	.48	.68	>.50	4.83	.38	6.22	.37	2.57	<.05
" creatinine N	1.15	.12	.39	.70	1.10	.03	1.38	.11	2.37	.05
" creatine N	.67	.12	.63	>.50	.78	.13	1.64	.21	3.56	<.01
" uric acid N	.32	.02	.13	.90	.31	.02	.30	.02	.32	>.70
Sum	44.50				59.60		109.00			
			p >.40						p >.10	
Total N as determined by micro-Kjeldahl	42.40				59.60		124.00			

* S.E. represents stand. error, p indicates level of probability, and t stands for critical ratio of the means.

consistency was found in the nitrogen excretion of the rats within each environmental group. The average values for each 50-day test period along with the standard error and critical ratio of the mean are set forth in Table I.

The diet used throughout contained 82.0% bleached flour, 6.35% hydrogenate oil (Crisco), 5.30% sodium caseinate, 4.25% total salt mix, 1.06% water soluble vitamins in sucrose, and 1.06% oil soluble vitamins (Sardilene oil). Fourteen kilograms of the diet were supplemented with 1.0 g of alpha-tocopherol and 6.50 g of choline. Total nitrogen determinations indicated that this diet contained 0.80% nitrogen. All animals were maintained on the ration for 3 weeks prior to the test period and permitted to eat *ad libitum.*

Results and discussion. Both the total nitrogen intake and total urinary nitrogen decrease with high and increase with low ambient temperature (Table I). If these data are plotted, the relationship to the environmental temperature appears to be logarithmic, thus casting doubt upon the proposal of Johnson and Kark(13) that the nitrogen intake, which is directly determined by the total food intake, would be a linear function of environmental temperature.

Comparing the total nitrogen consumed in the ration with that appearing in the urine we note that with rats maintained at either 23°C or 35°C the total nitrogen consumed in the ration exceeds that appearing in the urine. At 4°C, however, the nitrogen eliminated approximately equals that which is taken in with the food, suggesting that the 5% level of dietary protein at normal and high temperature was adequate for all metabolic needs plus considerable addition to body mass. At a low temperature, however, the cold stress appears to have made the availability of nitrogen a limiting factor for growth, despite the overall increase in food consumption.

Of the urinary constituents, urea best represents exogenous nitrogen metabolism. This component (Table I) rises sharply as the exposure temperature falls. This is to be expected in view of the increased food intake and the undoubted increase in protein metabolism occurring at lower temperatures. Even on a low nitrogen diet it is to be concluded that the animal utilizes a large fraction of its available protein, through deamination, to secure a maximal supply of energy.

With regard to those components derived predominantly from endogenous metabolism, there is a clear effect of exposure temperature on allantoin and creatine. Particularly at 4°C is there a marked increase in the excretion of these substances.

Since allantoin measures the breakdown of nucleic acids, it may be postulated that the reduced temperature actuates a rise in tissue metabolism which is associated with augmented oxidative metabolism of cell protein. Creatine excretion probably follows carbohydrate metabolism and the turnover of high energy esters such as creatine phosphate. The latter processes, of course, vary inversely with ambient temperature and may occur in muscles as well as other tissues.

The output of uric acid and creatinine is relatively unaffected by temperature. That of ammonia and amino acid nitrogen apparently increases at 35°C, but is unaffected by cold. We have no specific explanation for these findings.

On the whole it is reasonably clear that protein metabolism is influenced by prolonged exposure to high and low environmental temperature, and follows the increased carbohydrate utilization reported by Baker and Sellers(14) as well as the increased fat utilization described by Young and Cook(15) in cold treated rats. The critical requirement is the energy supply. Thus in the cold the total intake of protein increases in conformity with the food. Of the increased quantity, a greater proportion is converted to carbohydrate and burned rather than being utilized for growth. The increase in the metabolism of cell protein may also be associated with energy requirements.

Summary. Adult male rats were maintained on a low protein diet and exposed to 3 environmental temperatures, 4°C, 23°C, and 35°C, for 50 days. Measurements of total urinary nitrogen as well as food intake and changes in body weight were made every third day between the tenth and fiftieth day of exposure. Food intake, total urinary nitrogen, and the various urinary fractions, urea, allantoin, and creatine, were found to be inversely proportional to the exposure temperature. Excretion of uric acid, ammonia and amino acids, and creatinine bore no apparent relation to external temperature. The changes in urea, allantoin, and creatine excretion reported here are proportional to the metabolic stresses imposed by high and low temperatures.

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Effect of ACTH and Adrenocortical Hormones on Experimental Gas Gangrene Toxemia. (21853)

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The incidence of gas gangrene has decreased progressively in recent wars, due mainly to improvements in surgical organization, teaching, and technic, use of blood transfusion and availability of antibacterial agents. Fatal gas gangrene is still encountered following trauma even though the generally accepted methods of treatment are employed. It is important, therefore, to evaluate continually additional therapeutic methods which may be helpful when usual forms of treatment are inadequate. Recently, Haedicke and Glascock(1) reported a case of gas gangrene in which they felt that administration of adrenocorticotrophic hormone may have been beneficial. A survey of the literature revealed no experimental studies on the effect of ACTH or other adrenocorticosteroids on the toxemia of gas gangrene. The present paper presents the results of such a study.

Methods and materials. Female rats (Shenan strain) weighing 80 to 100 g were used and maintained on Purina Dog Chow and water. The effect of varying doses of the steroids to be tested was evaluated in groups of 10 rats, each of which was given one LD_{50} of *Clostridium perfringens* toxin. A filtrate of *Clostridium perfringens* type A was used.* This filtrate was analyzed for alpha toxin(2), theta toxin(3), collagenase(4) and hyaluronidase(5). The LD_{50} for the rat of this filtrate was determined following intramuscular injection. The LD_{50} was determined 3 times, using groups of 10 animals for each dilution. The following drugs were used: 1) Cortisone Acetate (Merck & Co.); 2) Zinc Adrenocorticotrophic hormone† (ACTH); 3) Hydrocortisone Acetate (Pfizer & Co.); 4) Lipo Adrenal Extract (Upjohn & Co.); and 5) Desoxycorticosterone Acetate† (DOCA). Dilutions of cortisone, ACTH, hydrocortisone, and DOCA were prepared with 0.9% sodium chloride. Lipo Adrenal extract was diluted with sterile cotton seed oil.‡ Throughout these experiments hormone preparations and toxin were administered intramuscularly.

TABLE I. Effect of Cortisone and of Hydrocortisone Injected 16 Hr Prior to and Again at Time of *Cl. perfringens* Toxin Injection. (Rat survival recorded 24 hr post-toxin injection.)*

Hormone, μg	Cortisone treated†	Hydrocortisone treated†
75	15/30	6/20
150	11/30	—
250	21/30	5/20
750	12/30	9/20
1000	13/30	11/20
2500	12/20	8/20
—	13/30	11/20
2500	30/30	20/20

* 0.4 ml of toxin was given for each group of rats used, except for last group.

† No. of rats surviving out of total in group.

Results. Using the Reed Munch Method, 24-hour LD_{50} equalled 0.42 ml of undiluted toxin. The 95% confidence limits, calculated on the least square regression line ranged from 0.32 to 0.50. Throughout the present study 0.4 ml of toxic filtrate was used as the test dose of toxin.

The filtrate of *Clostridium perfringens* toxin used in these studies revealed on analysis the following constituents: 128 units alpha toxin per ml; 50 units theta toxin per ml; 5 units collagenase per ml and 16 units hyaluronidase per ml.

Effect of administration of hormones 16 hr prior to and at time of injection of toxin. The 24-hour survival of groups of rats given cortisone, hydrocortisone, ACTH, lipo adrenal extract, or DOCA in addition to one LD_{50} of toxin is recorded in Tables I and II. Each hor-

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* Obtained through the courtesy of Lederle Laboratories, Pearl River, N. Y.

† Obtained through the courtesy of Organon, Inc., Orange, N. J.

‡ Preparation Res. No. 10,250 obtained through the courtesy of the Upjohn Co., Kalamazoo, Mich.

TABLE II. Effect of ACTH, Lipo Adrenal Extract (LPA) and of Desoxycorticosterone Acetate (DOCA) Injected 16 Hr prior to and Again at the Time of *Cl. perfringens* Toxin Injection. (Rat survival recorded 24 hr post-toxin injection.)*

Hormone, ml	ACTH treated†	LPA treated†	DOCA treated†
.001	—	6/20	—
.004	5/20	8/20	—
.01	10/20	8/20	10/20
.02	6/19	11/20	11/20
.04	9/20	—	3/20
.1	9/20	8/20	9/20
.2	7/20	11/20	10/20
—	7/20	11/20	8/20
.2	20/20	20/20	20/20

* 0.4 ml of toxin was given for each group of rats used, except for last group.

† No. rats surviving out of total in group.

mone was given 16 hours prior to and again at the time of toxin administration. A wide range of dosage was used for each agent tested. The tables also record the control data of rat survival following the injection of one LD_{50} of toxic filtrate in the absence of hormones. Similarly, in the absence of toxin, rat survival is recorded following the injection of the greatest dose of hormone used per experiment. No significant differences were apparent in the survival of hormone treated as compared with control animals receiving toxin alone. All animals receiving hormone alone survived. Animals receiving 2.5 mg of cortisone plus toxin appeared to show an increased survival rate in that 21 of 30 animals survived compared with 13 of 30 controls. These tests were done at different times with groups of 10 rats. In 2 of the 3 groups, 2.5 mg of cortisone produced no beneficial effects on survival rate. In the third experiment at that dosage level, survival rate was markedly improved. The figure reported in Table I of 21 of 30 rats surviving is the result of the 3 experiments and is not statistically significant.

TABLE III. Effect of Cortisone and of Hydrocortisone Injected Two Hr Prior to *Cl. perfringens* Toxin Injection. (Rat survival reported 24 hr post-toxin injection.)*

Hormone, μg	Cortisone treated†	Hydrocortisone treated†
100	7/10	0/10
250	4/10	1/10
500	4/10	2/10
1000	—	3/10
2500	4/10	4/10
—	5/10	2/10
2500	10/10	10/10

* 0.4 ml of toxin was given for each group of rats used, except for last group.

† No. rats surviving out of total in group.

Effect of administration of hormone 2 hr prior to toxin injection. Again, no significant differences were found where varying doses of test substance were used compared with survival after injection of one LD_{50} of toxic filtrate alone (Tables III and IV.)

Effect of administration of hormones at the time of toxin injection. Administration of varying doses of ACTH or of cortisone at the same time rats received one LD_{50} of toxic filtrate did not influence the 24-hour survival rate (Table V).

Discussion. The data here reported show that ACTH, cortisone, hydrocortisone, lipo adrenal extract, and DOCA were not beneficial in the treatment of experimental *Clostridium perfringens* toxemia. The hormones were tested over a wide dosage range and were administered both before and at the same time toxin was injected. These data suggest, therefore, that if a beneficial result is obtained following administration of ACTH or adrenocorticosteroids to a patient with gas gangrene, some nonspecific mechanism is involved. There is a difference of opinion as to the degree of injury of the adrenal cortex in gas gangrene as judged by histopathological study. Dietrich and Geringer(6,7) in World War I found hyperemia, foci of necrosis, disappearance of lipid substance, and leucocytic infiltration of the adrenals. Govan, and Fa-

TABLE IV. Effect of ACTH and of Lipo Adrenal Extract (LPA) Injected Two Hr Prior to *Cl. perfringens* Toxin Infection. (Rat survival reported 24 hr post-toxin injection.)*

Hormone, ml	ACTH treated†	LPA treated†
.001	—	5/10
.004	3/10	3/10
.01	2/10	4/10
.04	5/10	5/10
.1	2/10	2/ 9
—	3/10	4/10
.1	10/10	10/10

* 0.4 ml of toxin was given for each group of rats used, except for last group.

† No. rats surviving out of total in group.

TABLE V. Effect of Cortisone and of ACTH Administered at the Time *Cl. perfringens* Toxin was Injected. (Rat survival reported 24 hr post-toxin injection.)*

Cortisone		ACTH	
μg	Survival	ml	Survival
100	5/10	.004	4/20
250	6/10	.01	7/20
750	6/10	.02	7/20
2500	5/10	.1	6/20
—	6/10	—	7/20
2500	10/10	.1	20/20

* 0.4 ml of toxin was given for each group of rats used, except for last group.

vata(8,9) and associates found essentially normal adrenal glands in clinical and experimental gas gangrene. No physiological or chemical studies of adrenocortical function in gas gangrene have been found in the literature. Cardiovascular collapse is a manifestation of a progressive gas gangrene, but there is no clearcut evidence that this is the result of a failure of adrenal function. Favata and associates(9) have reported that 23 of 26 dogs with experimental gas gangrene showed histologic evidence of cardiac damage. Hayes has suggested that some patients in "irreversible" shock may have a temporary peripheral adrenocortical insufficiency because of hemodynamic failure(10). It is possible that a patient with gas gangrene and cardiovascular collapse might be so affected even though the adrenal cortex is not specifically damaged.

Experimental studies by others have shown that ACTH and adrenocorticosteroids will not protect against diphtheria, tetanus or Shiga toxin nor against meningococcal infection (11, 12). Therefore, failure to find an apparent antitoxic effect of ACTH or adrenocorticosteroids against experimental *Clostridium perfringens* intoxication in the present experiment is not surprising.

Summary. The effect of ACTH and several adrenal corticosteroids on experimental gas gangrene toxemia was studied. Treated rats were challenged with a standard dose of *Clostridium perfringens* type A filtrate. Results indicate that none of the above drugs increased the rate of survival.

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Oral Smear as a Method of Chromosomal Sex Detection.* (21854)

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The sexual dimorphism in nuclei of several human tissues has recently been described (1,2) and the principle has proved its usefulness in clinical application. Using the skin biopsy technic of Barr, several investigators have studied the genetic sex in cases of pseudohermaphroditism(3,4) and ovarian agenesis(5,6). These latter studies have revealed that the chromosomal sex in the majority of cases of "ovarian" agenesis is masculine, leading to the realization that "gonadal" agenesis is a more accurate designation of the clinical situation. It has been a natural trend to investigate methods which would avoid the operative procedure required for a skin biopsy. Davidson and Smith(7) have reported the appearance of a nuclear mass in neutrophils from females which they believe to be the sex chromatin. Thus, they find it possible to detect chromosomal sex by the means of a simple blood smear. We have found that cells scraped from the oral mucosa of females frequently contain sex chromatin masses in the nuclei while similar masses occur only rarely in males. On this basis chromosomal sex can be identified. The ease of obtaining oral smears and the facility of histological preparation offers distinct advantages for the clinical use of this technic.

Methods. Oral scrapings are thinly smeared on an ordinary microscope slide. A small metal spatula is suitable for obtaining material from an infant's buccal mucosa while the end of the slide has been adequate for an adult, using another slide to make the spread. It is very important that the slide should be immersed immediately while still moist in modified Davidson's solution(1). The slides may remain in the fixing solution from ½ hour to 24 hours. The preparations are stained according to a modified Feulgen technic(8) and counterstained with dilute light green (0.5% aqueous light green 5 parts and 80% alcohol 95 parts). The incidence of sex chromatin in a total of 100 nuclei from each smear is determined under oil immersion. In making these counts sex chromatin masses lying against or in close proximity to the nuclear membrane only were included.

TABLE I. Incidence of Sex Chromatin in Cells from the Oral Mucosa.

	No. cases	Age group	Range, %	Avg, %
Females	15	Newborns to 1 yr	20–68	44.2
	35	1 to 86 yr	22–79	46.1
Total	50	Newborns to 86 yr	20–79	45.6
Males	21	Newborns to 1 yr	0–4	.5
	29	1 to 69 yr	0–2.5	.6
Total	50	Newborns to 69 yr	0–4	.6

Results. Oral smears were obtained from 50 females (newborns to 86 years of age). The sex chromatin body was found in 20% to 79% of the nuclei, the average incidence being 45.6% (Table I). The sex chromatin, a plano-convex nuclear body in its characteristic position lying adjacent to the nuclear membrane is indicated by the arrow in Fig. 1 which should be compared with the nuclei in Fig. 2 representing the condition in males. In oral smears obtained from 50 males (newborns to 69 years of age) masses similar to sex chromatin were found in an average of 0.6% of the cells. The range of values obtained extended from 0%, which occurred frequently, to 4%. This range differed markedly from that obtained in female cases (Table I). In addition to the 100 cases with normal sex differentiation, we employed the oral smear technic in 2 cases of gonadal agenesis. Both smears gave cell counts characteristic of males.

Discussion. The efficiency of the oral

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smear technic herein described seems proved by our series of 100 cases since there were no overlapping values. The complete procedure, from obtaining the sample to the final interpretation of the smear, can be carried out in a matter of hours. We have noted an additional advantage to this method in that identification of the sex chromatin in the large mucosal cells is readily accomplished by the relatively inexperienced observer. Until the present time, only a few laboratories have had adequately trained personnel for the accurate interpretation of chromosomal sex from skin biopsies. It has been the experience of the authors that skin biopsies that are poorly fixed or prepared cannot be used for the interpretation. While poor preparation of an oral smear may also occur, the simplicity of securing a second sample is a decided advantage.

Summary. A method is described for detection of chromosomal sex from a smear of oral mucosa cells. In 50 females 20-79% of the cells had sex chromatin bodies in the nuclei. The occurrence of similar masses in oral smears from 50 males ranged from 0-4%. The method offers the advantage of ease in securing suitable cells, rapidity in preparation and simplicity of interpretation.

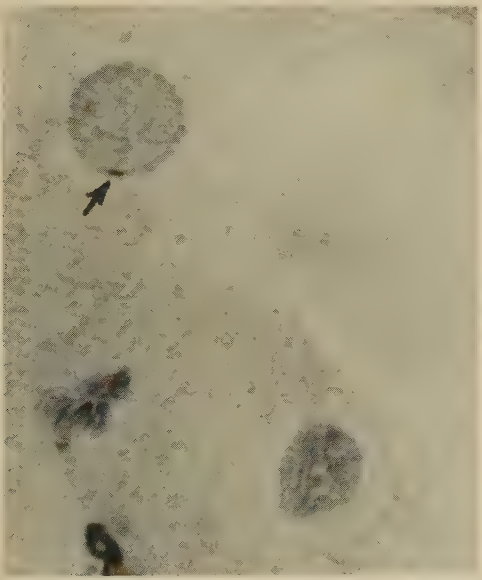

FIG. 1. Sex chromatin in its characteristic position in scraping from oral mucosa of an adult woman.

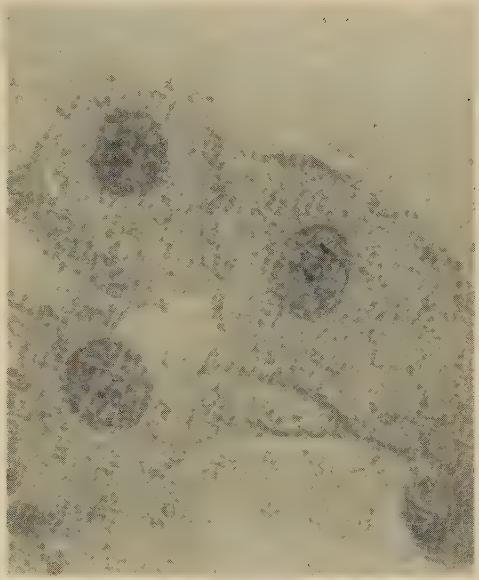

FIG. 2. Scraping from oral mucosa of an adult man. Note absence of sex chromatin.

We wish to thank Dr. J. T. Bradbury for his help in providing facilities for the collection of smears.

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Short Term Heat Stability of Poliomyelitis Vaccine. (21855)

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We have recently indicated the usefulness of a rapid immunity test in mice(1) of trivalent poliomyelitis vaccine prepared from all 3 strains of virus grown in monkey kidney tissue culture and inactivated with formalin as Salk has described(2). In these rapid tests single 0.5 ml doses of 5-fold dilutions of vaccine were injected into mice which developed active immunity in 3 or 4 days as evidenced by direct challenge with all 3 types of live virus in proper dosage. Such tests involving the type 2 vaccine component had been suggested originally by Salk and Krech and published by Krech(3).

These rapid mouse tests, along with other tests, have recently been used by us to solve the unanticipated cause of degradation of this vaccine by 'Merthiolate' (Thimerosal, Lilly) in the course of which it was found that the trisodium salt of ethylene diamine tetraacetic acid (EDTA) should be used to chelate the metallic residue in the vaccine (mainly copper) responsible for the catalytic oxidation of the preservative(4). When this is done it was found that full antigenicity of 'Merthiolate' preserved poliomyelitis vaccine was maintained after heating for 2 weeks at 37°C.

It was thought useful at this time to provide some added information on short term heat stability of the regular trivalent vaccine containing 'Merthiolate' 1:10,000 and EDTA 7-10,000(5) through use of the one dose mouse immunity test. The results of these tests are presented as follows:

Materials and methods. Trivalent poliomyelitis vaccine lot 649332 (the "filling" number) produced by the Biological Production Division of this company was used throughout these tests. This lot of vaccine is the same as the "bulk" lot 646783, the potency of which has been indicated(1).

Several ampoules of this vaccine were heated in a water bath at 40°C for 10, 20, and 30 minutes respectively, and also several for these times at higher temperatures including 5°C increments from 40°C up to and including 70°C. These 21 heated vaccines were injected, in a series of 5-fold dilutions, into groups of 10 mice which were challenged with the particular viruses already described(1). Non-heated vaccine from lot 649332 comprised a control vaccine administered to mice in every group of tests. Also, control mice for virus virulence were regularly included in every group of tests. We have assayed the response against the types 1 and 2 components of the vaccine on account of the well known greater lability of 1 compared to 2, and 2 as compared to 3.

Results. The results of these tests are indicated in Table I, and are recorded in terms of the number of mice, out of groups of 10 receiving different 5-fold dilutions of vaccine, which suffered no specific ill effects from the doses of challenge viruses. In a few instances, the groups were less than 10 mice and this is indicated in parenthesis. During the tests, all the vaccines were numbered in an alphabetical code, and it is noted that repeated use of unheated control vaccine and virus controls applied to certain groups of heated vaccines tested at a given time.

It is apparent that when the vaccine is heated at 40°C or 45°C for 10, 20, or 30 minutes, the types 1 and 2 components show good antigenic stability. When heated at 50°C, the type 1 component is weakened somewhat by 10 minutes' heating and more so by 20 or 30 minutes. When heated at 55°C both types 1 and 2 components are destroyed in 10, 20, or 30 minutes, and this is also true at heating temperatures of 60°C, 65°C, and 70°C.

It is apparent also that reasonable regularity of positive results (successful immunization) is shown in the vaccines heated at 40°C and 45°C as well as the control vaccine, while regularity of negative results (failure of immunization) is shown in the vaccines heated at 55°C, 60°C, 65°C, and 70°C.

TABLE I. Tests of Heated Poliomyelitis Vaccine in Mice.

Vaccine heated at:	Dil. of vaccine used to immunize mice	Vaccine heated for: 10 min. Type 1	10 min. Type 2	20 min. Type 1	20 min. Type 2	30 min. Type 1	30 min. Type 2
40°C	Str.	B 6	8	J 5	9	P 6	8
	1:5	8	5	10	9	5 (9)	9
	1:25	7	3	7	6	4	2
	1:125	3	7	6	4	4	3
	1:625	3	3	5	4	4	2
45	Str.	C 5	8 (9)	K 9	9	R 7 (8)	9
	1:5	5	7	6 (9)	6	8	7
	1:25	7	5	5	5	6	8
	1:125	4	6	5	5	5	0
	1:625	3	1	2	2	5	3
50	Str.	D 4	10	L 3 (9)	8	S 2	6
	1:5	2	8 (9)	2	6	2	5(9)
	1:25	3	4	1	7	1	4
	1:125	0	3	2	1	0	3
	1:625	1 (9)	1	2	0	2 (9)	2
55	Str.	E 1	2	M 0	1	T 1	2
	1:5	1	0	0	2	2	2
	1:25	1	2	1	2	0	1
	1:125	1 (9)	1	0	2	1	0
	1:625	0	1	3	0	0	2
60	Str.	F 2	1	N 1	1 (9)	V 1	0
	1:5	1	4	1	1	1	1
	1:25	1	1	1	1	1	2(9)
	1:125	1	1	1	2	2 (8)	1(9)
	1:625	0 (9)	1	1	2	2	2
65	Str.	G 0	3 (9)	O 1	2	W 1	1
	1:5	0 (9)	1	1	2 (9)	0	1
	1:25	1	0	1	1 (9)	2	2
	1:125	0	1	2	0	2	1
	1:625	1	2 (9)	0	1	1	2
70	Str.	I 0	1 (9)	Y 2	2	Z 1	2
	1:5	0	2	0	1	0	2
	1:25	0	3	0	1	0	1
	1:125	0	3	0 (8)	0	0	1
	1:625	0	1 (9)	1	2	1	0

Control vaccine 649332; not heated

		Type 1 for lots B-C-D-J K-L-P R-S	Type 2 for lots B-C-J-K P-R	Type 1 for lots E-F-G-M N-O-V T-W	Type 2 for lots D-E-L-M S-T	Type 1 for lots I-Y-Z	Type 2 for lots F-N-V-G	Type 2 for lots I-O-W Y-Z
Not heated	Str.	5	9	7	9	7 (9)	9	8
	1:5	7	9	8	4	5	7	2 (9)
	1:25	6	5	7	5	10	5	5
	1:125	5	4	4	4	6 (9)	2	1
	1:625	3	6	4	5	2	4	3 (9)
Virus controls								
1.0 challenge dose		1	3	1	1	0	0	2
.1 " "		3	8	5	8	9	7	6

Results are recorded in terms of mice living and normal out of groups of 10 used.

Usefulness of the one dose mouse immunization test is attested in this way, and the results need no particular interpretation.

Summary. Poliomyelitis vaccine as currently made is almost completely deprived of antigenicity when heated at 55°C for 10 to 30 minutes as shown by assays of response to types 1 and 2 components in white mice.

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Process of Healing in Experimental Pulmonary Arteriosclerosis.* (21856)

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Pulmonary vascular lesions can be induced in dogs by anastomosing a systemic artery into the pulmonary artery(1,2), thus increasing pulmonary blood pressure and flow. Within 2 weeks the pulmonary arterioles may show hypertrophy of the media, and in 2 or 3 months progressive obliteration of the lumen may appear, due to intimal proliferation. The changes resemble those seen with some types of pulmonary hypertension in the human. A preliminary study of the effect on the lesions produced by removing the shunt and reanastomosing the pulmonary artery to the proximal stump, in which the pressure was normal, showed little evidence of regression after a period of time equal to that required for changes to develop(3). The present study is a histologic appraisal of the further course of vascular changes in 3 dogs observed 14, 20, and 21 months after the shunt was removed. The pulmonary artery anastomoses were shown to be open at those times. The shunts had functioned 6 to 8 months, during which time severe lesions had developed (Fig. 1).

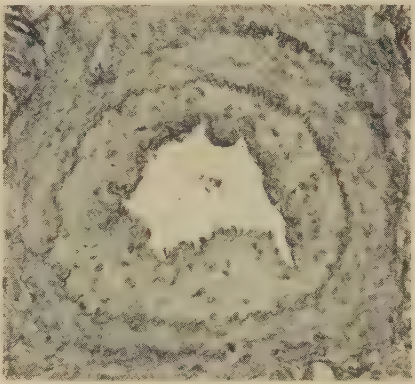

FIG. 1. Artery (230 μ) at time shunt removed. Collagenous thickening of adventitia, muscular medial hypertrophy, and proliferative intimal thickening are present.

Method. Lung biopsies were taken at thoracotomy from 2 or 3 areas, fixed in formalin, or in lead acetate-formalin for toluidine blue staining, embedded and sectioned in the usual manner, or frozen and sectioned for fat staining. Stains used included Verhoeff's and Van Gieson's, toluidine blue, Schiff periodic acid, and Sudan IV. Vessel measurements were made with a micrometer eyepiece, making an assumption that the external elastic lamina still represented the true size of the vessel after severe sclerosis was present. Over 100 arterioles or small arteries, from 20 to 500 μ in diameter of the external elastic lamina, were measured from each animal at each time.

Results. The sections provide evidence that reestablishment of vascular patency oc-

TABLE I. Percentage of Arteries with Normal Structure and with Changes Leaving the Lumen Diameter Greater or Less than One-Half of Normal.

	Biopsy at time shunt removed			Biopsy 14-21 mo later		
Dog No.	N	Lumen >½	Lumen <½	N	Lumen >½	Lumen <½
1	0	22	78	2	68	30
2	4	35	61	29	49	22
3	44	35	21	72	25	3

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* This study was supported by a research grant from the National Institutes of Health, Public Health Service, project H-1960.

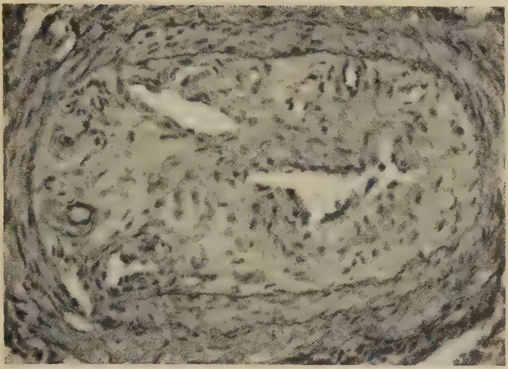

FIG. 2. Artery (220 μ smaller diameter) from same dog as Fig. 1, showing capillary and larger channels developing in thickened intima, 14 mo after shunt was removed. Media and adventitia are less prominent. Section is oblique.

curs at a slow rate (Table I). No sign of progression of lesions has been seen after shunt removal. The first definite change is the development of a more prominent capillary network within the proliferative intimal thickening (Fig. 2). Some of these channels continue to enlarge until the original lumen may be effectively restored (Fig. 3). In many vessels the cellularity of the intimal layer decreases and more collagen is visible, this process beginning near the intimal elastic lamina and progressing toward the lumen. Thickening of media and adventitia become less frequent and less pronounced though some vessels are apparently unchanged in these respects. Hydropic changes are sometimes found in the intimal layer. The process of recanalization as well as the hydropic changes may be seen in a few vessels, and to a small degree, while the shunts are still functioning. The type of change shown in Fig. 3, however, has not been found until more than 8 months after pulmonary artery anatomy has been restored to normal.

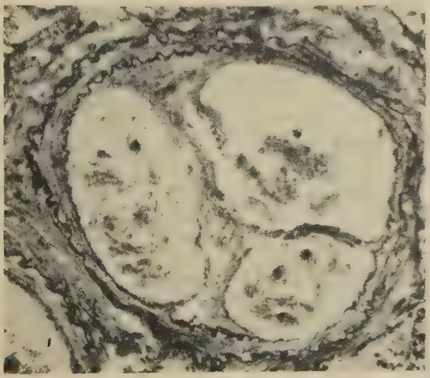

FIG. 3. Artery (270 μ) from same dog as Fig. 1 and 2, 14 mo after shunt was removed. Recanalization has largely restored the lumen.

Repeated studies have failed to show any sudanophile material in the vessels at any time. Most of the vessels with thick intimal lesions show little metachromasia with toluidine blue, but an occasional artery has shown this change to a more pronounced degree. Results with the Schiff periodic staining have also been negative, in that there is no evidence of any abnormal accumulation of carbohydrate in the damaged vessels.

Discussion. The effectiveness of surgery for cardiovascular anomalies associated with pulmonary hypertension, such as patent ductus arteriosus, sometimes depends on whether the pulmonary circulation can be maintained in effective use at lower pressures than those present before operation. It seems apparent from the present study that recovery from severe vascular lesions is likely to be prolonged, and that consequently the operative procedure might better be done in stages if such lesions are present. The experiment differs from the clinical situation after corrective surgery, in that the amount of blood transmitted by the diseased lobe may be very low without consequence to the dog, in which the other lung is intact. In the human, correction of a shunt may require the pulmonary circulation to operate at higher than normal pressure in order to force blood through the diseased vessels, and this fact may affect the rate at which lesions regress, or perhaps progress, after surgery.

What role if any is played by thrombosis in the early pathogenesis of the intimal thickening is problematical. Thrombi that are identifiable histologically are extremely rare, and the early changes in the sclerotic process —medial hypertrophy and a thin layer of intimal proliferation—do not suggest a thrombotic origin. Inflammatory changes have not been seen. Re-establishment of a lumen, however, follows a pattern analogous to recanalization of a thrombus.

The failure to identify fat or mucopolysaccharides in the lesions by staining technics differentiates these vessels from those seen with some other types of experimental arteriosclerosis(4). It seems apparent that mechanical factors play the important role in etiology in the present experiment. When the inciting mechanical factors are removed, progression of the process stops and healing begins.

Summary. Slow reestablishment of patency, chiefly by recanalization, has been demonstrated in experimentally induced pulmonary arteriolar lesions in dogs, after the inciting mechanical stress is removed.

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Latent Period in Monkey Kidney and HeLa Cell Monolayer Cultures Infected with Poliomyelitis Virus.* (21857)

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It has previously been shown that the fraction of an inoculum of poliomyelitis virus adsorbed to a monkey kidney monolayer culture is independent of the multiplicity of exposure over the large range of 2 x 10^3 plaque-forming units per cell to 2 x 10^{-2} PFU/cell(1).

In order better to evaluate studies on virus-cell interaction, further experiments were carried out to determine the amount of virus adsorbed by the host cells under various conditions during the period of adsorption. In the present work it was found that determinations of the latent period, *i.e.* the time after infection at which the first release of virus could be observed, permitted an evaluation of the effects of variations in the time allowed for adsorption, the multiplicity of exposure, the temperature and the pH during the adsorption period.

Materials and methods. Virus. Poliomyelitis virus, type 2, strain MEF-1, obtained from Dr. Jonas Salk in 1952, was passed several times in monkey kidney cells and 3-4 times in HeLa cells in this laboratory. The final virus preparation was stored at –60°C.

Cells. The method employed for trypsinization and cultivation of monkey kidney cells has been described(1). HeLa cells: Malignant human epithelial cells, strain HeLa, were received from Dr. J. T. Syverton in 1952 and had, at the time of the experiment, gone through approximately 100 passages in this laboratory. The strain of cells was maintained in a medium consisting of 40% human serum, 2% embryo extract and 58% Hanks balanced salt solution. The approximate number of cells per tube at the time of inoculation with the virus was determined as previously described(1).

Infectivity. Infectivity measurements of the virus were made partly by plaque assays on monkey kidney plates(2,3) and partly by the CPE-titration method on monkey kidney tube cultures(1) as specified under the experiments.

Experimental procedures. Monolayer cultures in test tubes containing approximately 10^5 cells per tube were washed with phosphate buffer solution or medium 199 and then inoculated with the virus sample concerned. For each virus dilution 2 tubes were inoculated, each with 1 ml. Unless otherwise specified the time of adsorption was 45 minutes. The supernatants were then drawn off and replaced by 1 ml medium 199 per tube. In the following period this procedure was repeated, first at intervals of 15 minutes, then at intervals of 30 minutes and from 4 hours after inoculation at intervals of one hour. All the experiments, except Exp. 4, were carried

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out in a 37°C warm incubator, so that cultures, media and all equipment were kept at constant temperature. In this way the repeated washing procedures did not interfere with the temperature conditions under which the cells were maintained and virus release from the infected cells took place.

All the samples of supernatants drawn off the cultures were stored in the refrigerator until the assays for infectivity could be performed by the CPE-titration technic; samples of the inocula were assayed by the plaque technic. The pH of all media, except in Exp. 2 was between 7.0 and 7.5.

Exp. 1 was designed for determining the latent period between infection and the first observable release of virus from monkey kidney cells and HeLa cells infected with poliomyelitis virus in various multiplicities of exposure. The average number of cells per culture was 1.0 x 10^5 for the monkey kidney cultures, 1.6 x 10^5 for the HeLa cell cultures.

The graphs of Fig. 1 show the data obtained from a series of experiments in which cultures of the 2 types of cells were inoculated with virus preparations in a series of 10-fold decreasing multiplicities of exposure. The range of multiplicities decreased from 280 PFU/cell to 0.028 PFU/cell for the monkey cultures, and from 70 PFU/cell to 0.007 PFU/cell for the HeLa cells. All the samples of supernatants were assayed for number of PFU/ml, and the curves in Fig. 1 represent the increase in virus contents of the supernatants due to the release of virus produced, starting at a basic level obtained by the removal of the inoculum in the preliminary washings. The latent period for the monkey kidney cultures is found to be between 3½ and 4 hours for the multiplicities of 280 and 28 PFU/cell whereas the first detectable release of virus occurs between 4 and 5 hours for multiplicities from 2.8 down to 0.028 PFU/cell. The HeLa cultures began to release virus somewhat more rapidly since the increase occurs between 3 and 3½ hours for multiplicities of 70 and 7 PFU/cell, and between 3½-4 hours for a multiplicity of only 0.7 PFU/cell.

The results of this experiment indicate that the time at which the first release of virus can be observed depends on the multiplicity of exposure. In lower multiplicities of exposure a longer time is needed before virus release can be detected in the supernatant. A comparison of monkey kidney cells and HeLa cells with respect to the effect of varying the multiplicity of exposure to virus, indicates that the HeLa cells release virus about one-half hour earlier than monkey kidney cells under the conditions of study. The amount of virus released within the first hours is also lower for low multiplicities of exposure than for higher multiplicities. A comparison of the titers of the 7-hour samples obtained from the HeLa cells shows that approximately 1000 times more virus was released by the culture infected with a multiplicity of exposure of 7 PFU/cell than by the culture infected with only 0.007 PFU/cell.

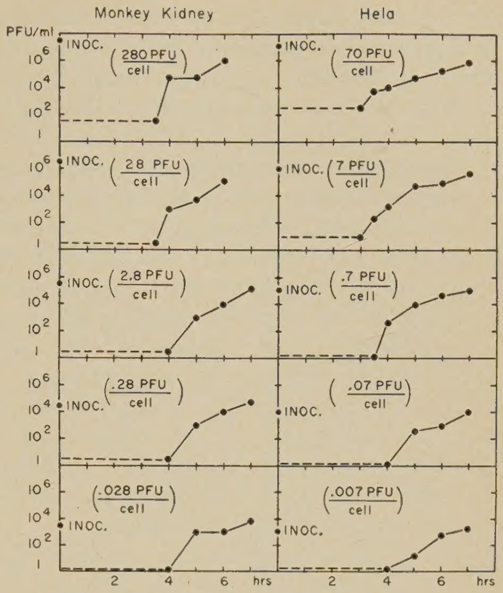

FIG. 1. Latent period and virus production with variation in multiplicity of exposure for monkey kidney and HeLa cells infected with poliomyelitis virus (MEF-1).

Exp. 2 was carried out in order to investigate the effect of variations of pH of the medium during the adsorption period. The virus preparation was diluted 1:10 with medium 199 and pH was adjusted to the values 6.0, 6.5, 7.0, 7.5, and 8.0 in a series of 5 samples. Parallel adsorption experiments were then carried out on monkey kidney cells with these samples by the technic already described.

After having allowed 45 minutes for adsorption the supernatants were drawn off and replaced by medium 199 of pH 7. All further washings were also done at pH 7. The multiplicity of exposure was 10 PFU/cell.

Results. The latent period was found to be 3½-4 hours independent of the pH in the adsorption medium. The amounts of virus released during the following hours did not vary significantly. Comparing this result with the results from Exp. 1 we can conclude that possible differences in the amount of virus adsorbed due to variations of pH between 6.0 and 8.0 do not materially affect the latent period or the amounts of virus released. For practical work it can therefore be assumed that small variations in pH during the adsorption period are of minor importance.

Exp. 3 was designed in order to study the effect of variations in the time allowed for adsorption of the virus to monkey kidney cells. This experiment was carried out with a multiplicity of exposure of 25 PFU/cell. The period of adsorption was respectively 5, 15, 30, 45 and 60 minutes in a series of 5 experiments.

Results. It was found that the latent period was 3½ to 4 hours and independent of the time allowed for adsorption when this time was 15 minutes or more. When only 5 minutes were allowed for adsorption the latent period was found to be 5-6 hours. The amounts of virus released during the first 7 hours were also found to be practically independent of the time allowed for adsorption when this time was at least 15 minutes. It appears that the main part of the virus that could adsorb in one hour did so within 15 minutes whereas the amount of virus that adsorbed within 5 minutes was considerably less.

Exp. 4 was carried out in order to study the effect of variations in temperature during adsorption and during virus production (monkey kidney cells). Three combinations of temperature were studied in this experiment:

	Temperature (°C) during:	
	Period of adsorption	Period of virus multiplication
A	37	37
B	20	37
C	37	20

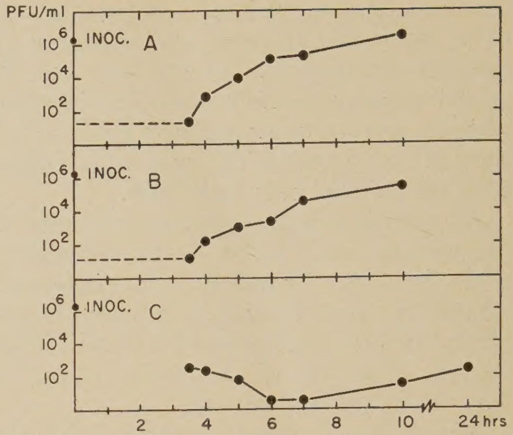

FIG. 2. Latent period and virus propagation with variations in temperature. A: Adsorption at 37°C, propagation at 37°C; B: Adsorption at 20°C, propagation at 37°C; C: Adsorption at 37°C, propagation at 20°C.

The time for adsorption was 45 minutes, the multiplicity of exposure 20 PFU/cell.

Fig. 2 shows the number of PFU/ml measured by the CPE-titration method in samples taken after 3½, 4, 5, 6, 7 and 10 hours for Exp. A, B and C and a further 24-hour period for Exp. C. The release of virus in both Exp. A and B begins 3½-4 hours after infection. The amounts of virus released are reduced when the temperature during the period of adsorption had been 20°C as compared to 37°C. This difference could be due to a lower rate of adsorption at 20° but might also be due to an effect of the 45 minutes period at 20°C on the state of the cells. In Exp. C even the sample containing the virus recovered during the whole period of time from 10 to 24 hours after infection shows only about 100 PFU/ml. It seems reasonable to conclude that the cells have not been able to produce virus at 20°C during this 24-hour period. Since the amount of virus recovered in the supernatants by repeated washings was far less than the amount inoculated in this experiment the cells must have adsorbed virus which could not be removed by washing.

Discussion. In order to determine the latent period for the very first release of virus by a cell culture it was necessary to be able to detect small amounts of virus and to avoid the masking effect of unadsorbed parental virus. The technic used consisted of a repeated removal and replacement of the medium at constant temperature followed by a titration of all the withdrawn samples. The washing procedure removed unadsorbed parental virus leaving a period during which only very small amounts of virus could be detected followed by a period during which the titer again started to increase due to the release of progeny virus.

From experiment to experiment the latent periods under the same experimental conditions came out identically, incidentally showing the practical value of the CPE-titration method. The 3½-4 hours latent period for monkey kidney cultures has been recorded 15 times. The variations in latent period due to variations of the multiplicity of exposure can be most reasonably explained by the assumption that the individual cells of the population in a culture release virus at slightly different times after exposure. When all cells are initially infected the release of virus from the most rapid cells is therefore detected. This theory is supported by the data of Lwoff *et al.*(4) indicating that the period of release of virus from a single cell is only 1-2 hours. Even if it is evident that some of the progeny virus will be adsorbed to the culture, a certain fraction will remain unadsorbed; the times recorded for the first increase in virus titer must therefore represent the actual time for the first release of virus from the culture. Monkey kidney cell cultures contain a fraction of cells which are able to produce and release virus 3½-4 hours after infection, and HeLa cell cultures contain cells which release virus after 3-3½ hours under our experimental conditions.

The experiments in which the pH during the adsorption period is varied can give only a rough indication of the effect of pH on the adsorption. It seems that variations in pH between practical limits during the adsorption period are unimportant.

The time allowed for adsorption is important. When less than 15 minutes are allowed the amount of virus adsorbed is below the optimum. In daily work it must be recommended to use at least 30 minutes for adsorption.

The temperature dependence for adsorption and propagation of polio virus is comparable to data reported on bacterial viruses for which it is found that the rate at which bacterial virus adsorbs to bacteria is decreased by a decrease in temperature(5), and that the rate of virus production by *E. coli* B is greatly reduced by lowering the temperature from 37°C to 20°C(6).

Summary. By repeated replacement of the supernatant fluid in a culture of monkey kidney cells or HeLa cells inoculated with poliomyelitis virus, non-adsorbed parental virus can be removed, and the earliest release of progeny virus can be measured. Under the conditions described a constant latent period of 3½-4 hours was obtained for monkey kidney cultures after multiplicities of exposure ranging from 280-28 PFU/cell and for HeLa cultures after multiplicities of exposure of 0.7 PFU/cell. In the monkey kidney cells lower multiplicities of exposure increased the latent period to 4-5 hours, and in HeLa cells higher multiplicities of exposure reduced the latent period to 3-3½ hours. A variation of pH during the adsorption period between the values 6.0-8.0 did not perceptibly affect the latent period or the amount of virus released. The length of time allowed for adsorption, when greater than 15 minutes, did not affect the latent period or the amount of virus released. When only 5 minutes were allowed, the latent period was lengthened and the yield was diminished. The lowering of temperature during adsorption from 37°C to 20°C diminished the yield of virus. Virus propagation at 20° was negligible.

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